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Cytotoxic T lymphocytes (CTL) play an important role in eradicating tumor cells. The purpose of this project is to determine whether fusion peptides composed of insertion signal sequences and peptides derived from the breast cancer antigen HER2/neu can improve antigen presentation and induce antitumor CTL with higher efficiency against breast cancer. We demonstrated that the addition of synthetic signal sequence at the NH_2-terminus, but not at the COOH-terminus, of the HER2/neu epitopes greatly enhanced their presentation in antigen processing-deficient cells, breast cancer cells and dendritic cells. Importantly, peptide constructs, composed of the HER2/neu epitopes replacing the hydrophobic part of the signal sequences were the most effective. The efficiency of the signal sequences in facilitating the HER2/neu peptide presentation was confirmed also by using IFN-g release assays. We also studied the mechanisms involved in the enhancement of antigen presentation by the fusion peptides, and proved that the effective presentation of the loaded peptide constructs is a result of their efficient loading into the cytosol and not simple binding to the surface HLA molecules. These findings may be of practical significance for the development of synthetic anticancer vaccines and *in vitro* immunization of CTL for adoptive immunotherapy.

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INTRODUCTION

INDUCTION OF CYTOTOXIC T LYMPHOCYTES FOR IMMUNOTHERAPY OF BREAST CANCER (DAMD17-00-1-0184)

Cytotoxic T lymphocytes (CTL) play an important role in eradicating tumor cells. CTL recognize short fragments of intracellular antigens, 8-10 amino acids in length, complexed with MHC class I molecules. Cytosolic peptides are transported across the endoplasmic reticulum (ER) membrane with the help of the ATP-dependent transporters associated with antigen processing (TAP). Peptides complexed with class I molecules in the ER are then transported to the cell surface for recognition by CTL. The transport into the ER can also be accomplished via insertion signal sequences located at the NH_2-terminus of the precursor molecules. **Our working hypothesis is that fusion peptides composed of insertion signal sequences and human tumor-associated peptide antigens can improve antigen presentation and induce antitumor CTL with higher efficiency. In the case of the breast cancer antigen HER2/neu, this could amount to a more potent CTL induction and anti-tumor immunity against breast cancer.** The specific aims of this study are to construct fusion peptides composed of natural or artificial signal sequences and HER2/neu-derived minimal peptides and to test the effectiveness of the fusion peptides in TAP-deficient cells, breast cancer cells and dendritic cells (DC). We determined the efficiency of the *in vitro* immunization of human CTL using peptide-loaded DC. We also studied the mechanisms involved in the enhancement of antigen presentation by the fusion peptides. The use of synthetic fusion peptides with signal sequences for eliciting CTL responses against specific cell-surface tumor antigens offers a new approach to immunotherapy for breast cancers that do not respond to existing forms of treatment. Immunization with such fusion peptides may be used both for prevention and for treatment of breast cancers expressing specific tumor antigens. As more specific tumor antigens are revealed, this approach may provide a model for development of more effective vaccines for breast cancer and other tumors. In addition to vaccine development, this proposal is relevant to understanding the mechanisms of antigen processing and presentation by MHC class I molecules.

BODY

INDUCTION OF CYTOTOXIC T LYMPHOCYTES FOR IMMUNOTHERAPY OF BREAST CANCER (DAMD17-00-1-0184)

Task 1. To construct fusion peptides composed of natural or artificial signal sequences and HER2/neu-derived minimal peptides (months 1-6).

The HER2/neu proto-oncogene, expressed in breast cancer and other human cancers, encodes a tyrosine kinase with homology to epidermal growth factor receptor (1). HER2/neu protein is a receptor-like transmembrane protein comprising a large cysteine-rich extracellular domain that functions in ligand binding, a short transmembrane domain, and a small cytoplasmic domain (1). HER2/neu is amplified and expressed in many human cancers, largely adenocarcinomas of breast, ovary, colon, and lung. In breast cancer, HER2/neu overexpression is associated with aggressive disease and is an independent predictor of poor prognosis (2) (3). HER2/neu is considered a possible target for T-cell-mediated immunotherapy for several reasons: (i) the protein is large (1255 amino acids), contains epitopes appropriate for binding to most MHC molecules and thus is potentially recognizable by all individuals; (ii) HER2/neu is greatly overexpressed on malignant cells and thus T-cell therapy may be selective with minimal toxicity; (iii) the oncogenic protein is intimately associated with the malignant phenotype and with the aggressiveness of the malignancy, especially in breast and ovarian carcinomas (2) (3) (4).

Several class I-restricted HER2/neu-derived peptides recognized by breast and ovarian cancer-specific CTL have been described (Table 1 on page 19).

We utilized these T cell epitopes to construct fusion peptides with natural or artificial signal sequences. We compared the effectiveness of the following signal sequences in improving the antigen presentation: a) one from early region 3 of the adenovirus type 2 (5), b) one from IFN-β (6) and c) several artificial sequences, generated according to the structure and the distribution frequency of the amino acids in the natural signal sequences. Since the hydrophobicity of the fusion peptides is higher than that of the minimal peptide, we used a set of control fusion peptides with signal sequences situated on the carboxy-terminus of the minimal peptides. Thus, we were able to determine if an improved immune response generated with fusion peptides is due only to the higher hydrophobicity of the fusion peptide, or it is related to a better translocation of the minimal peptide through the ER-membrane. Since signal sequences do not contain specific amino acid residues other than a hydrophobic region of about eight residues, we tested whether replacing this region with the hydrophobic HER2/neu-derived peptides would result in a more efficient presentation of these epitopes. To study the generality of the signal sequence approach we designed similar constructs utilizing several HER2/neu-derived peptides. The amino-acid sequences of the synthetic peptide constructs utilizing the epitopes $HER2/neu_{48-56}$, $HER2/neu_{369-377}$, $HER2/neu_{654-662}$, and $HER2/neu_{789-797}$ are shown in tables 2-5.

Task 2. To test the effectiveness of the fusion peptides using TAP-deficient T2 cells (months 7-12).

To probe class I presentation of cells loaded with the fusion peptides and their counterpart minimal peptides we generated CTL recognizing the HER2/neu-derived peptides. We immunized *in vitro* peripheral blood mononuclear cells (PBMCs) from normal donors with these peptides in the presence of interleukin 2 and interleukin 7 using the following technique:

PBMCs were separated by centrifugation on Ficoll-Hypaque gradients and plated in 24-well plates at 5 x 10^5 cells/ml per well in RPMI medium 1640 supplemented with 10% human AB^+ serum, L-glutamine, and antibiotics. Autologous PBMC (stimulators) were pulsed with the HER2/neu synthetic peptides (10 μg/ml) for 3 h at 37°C. Cells were then irradiated at 3,000 rads, washed once, and added to the responder cells at a responder to stimulator ratio ranging between 1:1 and 1:4. The next day, 12 units/ml IL-2 (Chiron) and 30 units/ml IL-7 (R & D Systems) were added to the cultures. Lymphocytes were restimulated weekly with peptide-pulsed autologous adherent cells as follows: First, autologous PBMC were incubated with HER2/neu peptide (10 μg/ml) for 3 h at 37°C. Non-adherent cells were then removed by a gentle wash and the adherent cells were incubated with fresh medium containing the HER2/neu peptide (10 μg/ml) for an additional 3 h at 37°C. Second, responder cells from a previous stimulation cycle were harvested, washed, and added to the peptide-pulsed adherent cells at a concentration of 5 x 10^5 cells/ml (2 ml/well) in medium without peptide. Recombinant IL-2 and IL-7 were added to the cultures the next day.

The induction of CTL in human PBMC was monitored in a conventional ^{51}Cr-labeling release assay. Briefly, peptide-pulsed TAP^{-}/HLA-$A2.1^+$ human T2 cells were incubated with 10 μg of HER2/neu peptides or the MART-1 control peptide for 90 min during labeling with ^{51}Cr. After washing, the target cells were added to serially diluted effectors in 96-well microplates. After a 6-h incubation at 37°C, supernatants were harvested and counted in a gamma counter. Results are expressed as the percentage of specific lysis and determined as follows: [(experimental cpm--spontaneous cpm)/(maximum cpm--spontaneous cpm)] x 100. (Table 6).

Importance of the position of signal sequences

Peptide-loaded or pulsed T2 cells were tested for their ability to present HER2/neu peptides at different periods of time after loading or pulsing. T2 cells loaded with most of the constructs composed of signal sequence at the amino-terminus of HER2/neu peptides were recognized by CTL up to eight days after loading (Figures 1-3, left column). In contrast, constructs with carboxy-terminal position of the signal sequence were not efficient, even when ^{51}Cr-release assays were performed immediately after loading. This recognition was not due to surface binding of these constructs since pulsing of T2 cells with any of the constructs was not efficient (Figure 1-4, right column). Loading or pulsing with the minimal HER2/neu peptides resulted in a significant recognition and lysis of T2 cells for only one day after loading or pulsing, followed by a rapid decrease of recognition on day 3 and complete lack of recognition on days 5 and 8 after loading or pulsing. This finding suggests that the recognition of T2 cells resulted from simple binding of the HER2/neu peptides to surface HLA molecules, from which it rapidly dissociated. T2 cells loaded with the constructs composed of signal sequence at the amino-terminus of the peptide HER2/$neu_{789-797}$ were recognized by CTL up to three days after loading (Figure 4, left column).

Signal sequences containing HER2/neu peptides

Since signal sequences do not contain specific amino acid residues other than a hydrophobic region of about eight residues, we tested whether replacing this region with the hydrophobic HER2/neu peptides would result in a more efficient presentation of these epitopes (Figures 5-8). We found that one of the two constructs of this type (HER-IN-AF) was the most efficient in facilitating the HER2/neu peptide presentation. Eight days after loading with the construct $HER_{369-377}$-IN-AF, T2 cells were still lysed with more than 60% specific ^{51}Cr-

release (Figure 6). The constructs HER_{48-56}-IN-AF and $HER_{654-662}$-IN-AF were also effective (Figures 5 and 7). The second construct of this type (HER-IN-ES), although not as effective as HER-IN-AF, was able to facilitate the recognition of T2 cells (Figures 5-7). Pulsing of T2 cells with these constructs did not resulted in efficient presentation. Again, as in the first group of experiments, loading or pulsing with the minimal HER2/neu peptides resulted in recognition of T2 cells for only a short period of time.

In IFN-γ release assays, 10^5 HER2/neu-specidic CTL were coincubated with 10^5 peptide-loaded T2 cells for 20 hours at 37°C. The concentration of human IFN-γ in cocultured supernatants was then determined by ELISA as described previously (7). The results of the ELISA experiments are shown in table 7 A-D. These findings are in parallel with the ^{51}Cr-release experiments, and confirm that the most efficient constructs in facilitating the HER2/neu peptide presentation are the constructs of the type HER-IN-AF. As in the ^{51}Cr-release experiments, the constructs with the peptides $HER_{369-377}$ and $HER_{654-662}$ were the most efficient, while the constructs with the peptide $HER_{789-797}$ were the least efficient, especially on days 5 and 8 after peptide loading.

Task 3. To determine if the insertion signal sequences can enhance presentation of class I-restricted tumor-associated antigens in human tumor cells (months 13-18)

During the second year of the grant period we tested whether the most effective signal sequences, already selected in the experiments with the TAP-deficient T2 cells, can also improve HER2/neu antigen presentation in human breast cancer cells.

In this series of studies we used the HLA-A2+ human breast cancer cell line MCF-7 expressing high levels of HER2/neu and the cell line MDA-MB-231 expressing only basal levels of HER2/neu. HER2/$neu_{369-377}$-specific CTL and HER2/$neu_{654-662}$-specific CTL failed to recognize the breast cancer cell line MDA-MB-231, although the same effectors specifically recognized T2 cells pulsed with HER2/$neu_{369-377}$, HER2/$neu_{654-662}$ and the cell line MCF7 expressing HER2/neu (Table 8). Thus, we concluded that MDA-MB-231 cells do not express HER2/$neu_{369-377}$ and HER2/$neu_{654-662}$, and that this cell line was appropriate for our peptide-loading experiments.

We tested by ^{51}Cr-release assay if the low HER2/neu-expressing breast cancer cells MDA-MB-231 can be recognized more efficiently by the HER2/neu-specific CTL after loading with the fusion peptides. We also determined by ELISA if the peptide-loaded breast cancer cells can induce release of IFN-γ by the HER2/neu-specific CTL.

The lysis of the tumor cells by the HER2/neu-specific CTL was monitored in a conventional ^{51}Cr-labeling release assay. Briefly, peptide-loaded tumor cells were added to serially diluted effectors in 96-well microplates. After a 6-h incubation at 37°C, supernatants were harvested and counted in a gamma counter. Results are expressed as the percentage of specific lysis and determined as follows: [(experimental cpm--spontaneous cpm)/(maximum cpm--spontaneous cpm)] x 100.

Peptide-loaded breast cancer cells MDA-MB-231 were tested for their ability to present HER2/neu peptides at different periods of time after loading or pulsing. Tumor cells loaded with the constructs composed of signal sequence at the amino-terminus of the peptides were recognized by CTL up to eight days after loading (Figures 9-10, left column). In contrast, constructs with carboxy-terminal position of the signal sequence were not efficient, even when ^{51}Cr-release assays were performed immediately after loading. This recognition was not due to surface binding of these constructs since pulsing of the tumor cells with any of the constructs was not efficient (Figure 9-10, right column). Loading or pulsing with the minimal HER2/neu peptides resulted in a significant recognition and lysis of the tumor cells for only one day after loading or pulsing, followed by a rapid decrease of recognition on day 3 and complete lack of recognition on days 5 and 8 after loading or pulsing. This

finding suggests that the recognition of the tumor cells resulted from simple binding of the HER2/neu peptides to surface HLA molecules, from which it rapidly dissociated.

Since signal sequences do not contain specific amino acid residues other than a hydrophobic region of about eight residues, we tested whether replacing this region with the hydrophobic HER2/neu peptides would result in a more efficient presentation of these epitopes (Figures 11-12). We found that one of the two constructs of this type (HER-IN-AF) was the most efficient in facilitating the HER2/neu peptide presentation. Eight days after loading with the construct $HER_{369-377}$-IN-AF, the tumor cells were still lysed with more than 60% specific ^{51}Cr-release (Figure 11). The construct $HER_{654-662}$-IN-AF was also effective (Figure 12). The second construct of this type (HER-IN-ES), although not as effective as HER-IN-AF, was able to facilitate the recognition of the tumor cells (Figures 11-12). Pulsing of the tumor cells with these constructs did not resulted in efficient presentation. Again, as in the first group of experiments, loading or pulsing with the minimal HER2/neu peptides resulted in recognition of the tumor cells for only a short period of time.

In IFN-γ release assays, 10^5 HER2/neu-specific CTL were coincubated with 10^5 peptide-loaded tumor cells for 20 hours at 37°C. The concentration of human IFN-γ in cocultured supernatants was then determined by ELISA as described previously (7). The results of the ELISA experiments are shown in table 9 A-D. These findings are in parallel with the ^{51}Cr-release experiments, and confirm that the most efficient constructs in facilitating the HER2/neu peptide presentation are the constructs of the type HER-IN-AF.

Task 4. To identify of the mechanisms involved in the enhancement of antigen presentation by the fusion peptides (months 19-24)

The goal of this set of experiments was to prove that the effective presentation of the loaded peptide constructs is a result of their efficient loading into the cytosol and not simple binding to the surface HLA molecules. We also studied the role of TAP in class I presentation in human cancer cells, and tested the efficiency of different signal peptides in cancer cells with different levels of TAP expression.

a) Probing the mechanisms of peptide loading

To distinguish between loading of the peptides into the cytosol and simple binding of these peptides to the surface MHC molecules we used several approaches:

First, we removed β_2-microglobulin from the surface of peptide-loaded tumor cells by acid stripping. We found that acid-stripping solution with pH=3.5 was most efficient in decreasing the specific recognition of peptide-loaded cells.

Second, we used pronase for complete enzymatic digestion of HLA molecules on the cell surface after loading in order to be able to detect the appearance of new internally formed HLA-peptide complexes on the cell surface, but not pulsing of the cells.

Third, we used Brefeldin A (BFA), a metabolite of the fungus *Eupenicillium brefeldianum*, which specifically blocks protein transport from the ER to Golgi apparatus.

We found that Brefeldin A specifically blocks the recognition of the peptide-loaded tumor cells by the HER2/neu-specific CTL. In contrast, the acid-stripping and the treatment with pronase was not able to block antigen recognition for more than 24 hours (Table 10). These experiments confirmed that the antigenic peptides were introduced into the cytosol of the cells, resulting in a prolonged and more efficient antigen presentation.

b) Inducing a functional blockade of TAP by ICP47

Another aspect in these studies was to determine the mechanisms of enhancement of the antigen presentation by the fusion peptides in human tumor cell lines. Therefore, we developed a new test system utilizing the Herpes Simplex virus (HSV) protein ICP47. ICP47 is a cytoplasmic protein, which interferes with antigen presentation by physically associating with TAP within the cell and inhibiting peptide transport across the ER-membrane. By transfecting the ICP47 gene into several cancer cell lines we were able to generate a novel system for screening different fusion peptides for TAP-independent translocation of peptide antigens through the ER-membrane.

We transfected the breast cancer cell line MCF7 with ICP47, and observed permanent block of the function of TAP, and therefore lack of recognition of these cells by the CTL, which normally recognize and kill them. To select the sequences most effective in translocation of antigenic peptides across the ER-membrane of the breast cancer cells, we loaded the ICP47-transfected cells with several fusion peptides with different signal sequences. We then detected the expression of these antigens by ^{51}Cr-release assays. We found that only the most efficient peptide constructs - $HER_{369-377}$-IN-AF and $HER_{654-662}$-IN-AF - were able to restore the antigen presentation in the ICP47-transfected breast cancer cells (Table 11). This confirms that the signal sequence approach is very effective in improving antigen presentation, even in tumor cells with deficiency of antigen processing/presentation.

Task 5. To test if loading of dendritic cells with fusion peptides can enhance the presentation of tumor-associated antigens to CTL (months 25-33)

During the third year of the grant period we tested whether the most effective signal sequences can also improve and prolong HER2/neu antigen presentation in human dendritic cells.

In this series of studies we used dendritic cells (DC) isolated from peripheral blood of HLA-A2+ healthy donors. DC were cultured in RPMI supplemented with 10% FCS, 50 ng/ml human GM-CSF and 1000 IU/ml human IL-4. DC were loaded with the fusion peptides after 7 to 15 days of culture.

We loaded the HLA-$A2^+$ human dendritic cells with the most effective signal sequences selected in the experiments with the TAP-deficient 2 cells and the HLA-A2+ breast cancer cells. We found that the constructs $HER_{369-377}$-IN-AF and $HER_{654-662}$-IN-AF are the most efficient (Figures 13 and 14). The construct $HER_{369-377}$-IN-AF was more efficient than $HER_{654-662}$-IN-AF as long as 6 days after loading (Figure 13). DC loaded with the construct $HER_{654-662}$-IN-AF also presented $HER_{654-662}$ very efficiently (Figure 14). DC pulsed with these constructs were not recognized by the CTL. Loading or pulsing with the minimal HER2/neu peptides resulted in a significant recognition and lysis of the DC for only one day after loading or pulsing, followed by a rapid decrease of recognition on day 3 and complete lack of recognition on day 6 after loading or pulsing. This finding suggests that the recognition of the tumor cells resulted from simple binding of the HER2/neu peptides to surface HLA molecules, from which they rapidly dissociated. This data indicate that the signal sequences enhanced and prolonged the presentation of the HER2/neu-derived peptides by human DC.

To address the question whether prolonged presentation of the HER2/neu peptides actually improves the generation of cytotoxic T cells, we initiated *in vitro* cultures of peptide-loaded DC with responder T lymphocytes. Mononuclear cells from HLA-$A2^+$ healthy donors were cultured in RPMI-1640 supplemented with 10% human AB^+ serum, 10 ng/ml IL-7 and 12 IU/ml IL-2 in the presence of peptide-loaded or peptide-pulsed and irradiated (3,000 rad) DC. Cultures were re-stimulated weekly with peptide-loaded and irradiated DC. A key aspect of these studies was to determine if loading with fusion peptides can reduce the number of

weekly re-stimulations necessary to induce anti-tumor CTL activity. We found that T2 cells pulsed with $HER_{369-377}$ were lysed very efficiently by CTL generated with DC loaded with the construct $HER_{369-377}$-IN-AF (Figure 15). $HER_{654-662}$-IN-AF - loaded DC were also able to generate efficient CTL after two rounds of re-stimulation (Figure 16). In contrast, DC loaded with the minimal peptides $HER_{369-377}$ and $HER_{654-662}$ were not able to generate cytotoxic T cells. These results demonstrate that prolonged presentation of the HER2/neu-derived peptides on DC is actually associated with more efficient generation of CTL.

We investigated by ELISPOT whether the peptide-loaded DC can improve the priming of CTL by increasing the number of antigen-specific, IFNγ-producing cells (Table 12 A-B). CTL in each of the three groups showed similar reactivity with non-pulsed T2 cells. In contrast, pulsing of the T2 cells with $HER_{369-377}$ or $HER_{654-662}$ led to an enhanced spot production by CTL elicited with DC loaded with $HER_{369-377}$-IN-AF or $HER_{654-662}$-IN-AF, but not by CTL stimulated with non-loaded DC. CTL induced with DC loaded with the minimal HER2/neu peptides demonstrated only a marginal enhancement of spot production.

These studies suggest that with fusion peptide-loaded DC it is possible to enhance antigen-presentation and stimulation of cytotoxic T lymphocytes. This approach may facilitate the development of synthetic peptide vaccines for human cancer.

Task 6. Analysis of results for significance (months 34-36):

We analyzed the effectiveness of the fusion peptides in inducing HER2/neu-specific CTL as follows:

T2 cells, tumor cells and DC were loaded with the fusion peptides or corresponding minimal peptides. Values obtained from the ^{51}Cr-release assays were compared for statistical significance using a **paired two-tailed *t* test** (GraphPad Prism Software).

LOADING OF T2 CELLS

The statistical analysis showed that nine of the sixteen fusion peptides loaded on T2 cells enhanced the HER2/neu presentation significantly, as compared to the corresponding minimal peptides:

$HER2/neu_{48-56}$ - DERIVED FUSION PEPTIDES

MINIMAL PEPTIDE	FUSION PEPTIDE	t RATIO	P VALUE	STATISTICALLY SIGNIFICANT (P<0.05)
$HER2/neu_{48-56}$	ES-HER_{48-56}	4.033	0.0069	YES
$HER2/neu_{48-56}$	IS-HER_{48-56}	4.205	0.0057	YES
$HER2/neu_{48-56}$	HER_{48-56}-IN-ES	2.048	0.0865	NO
$HER2/neu_{48-56}$	HER_{48-56}-IN-AF	1.916	0.1039	NO

HER2/neu$_{369-377}$- DERIVED FUSION PEPTIDES

MINIMAL PEPTIDE	FUSION PEPTIDE	t RATIO	P VALUE	STATISTICALLY SIGNIFICANT (P<0.05)
HER2/neu$_{369-377}$	ES-HER$_{369-377}$	5.053	0.0023	YES
HER2/neu$_{369-377}$	IS-HER$_{369-377}$	4.765	0.0031	YES
HER2/neu$_{369-377}$	HER$_{369-377}$-IN-ES	2.525	0.0450	YES
HER2/neu$_{369-377}$	HER$_{369-377}$-IN-AF	3.987	0.0072	YES

HER2/neu$_{645-662}$

MINIMAL PEPTIDE	FUSION PEPTIDE	t RATIO	P VALUE	STATISTICALLY SIGNIFICANT (P<0.05)
HER2/neu$_{654-662}$	ES-HER$_{654-662}$	3.187	0.0189	YES
HER2/neu$_{654-662}$	IS-HER$_{654-662}$	2.403	0.0531	NO
HER2/neu$_{654-662}$	HER$_{654-662}$-IN-ES	2.942	0.0259	YES
HER2/neu$_{654-662}$	HER$_{654-662}$-IN-AF	3.206	0.0185	YES

HER2/neu$_{789-797}$

MINIMAL PEPTIDE	FUSION PEPTIDE	t RATIO	P VALUE	STATISTICALLY SIGNIFICANT (P<0.05)
HER2/neu$_{789-797}$	ES-HER$_{789-797}$	0.2132	0.8382	NO
HER2/neu$_{789-797}$	IS-HER$_{789-797}$	1.379	0.2172	NO
HER2/neu$_{789-797}$	HER$_{789-797}$-IN-ES	1.759	0.1291	NO
HER2/neu$_{789-797}$	HER$_{789-797}$-IN-AF	1.922	0.1030	NO

LOADING OF BREAST CANCER CELLS

All of the fusion peptides loaded on breast cancer cells enhanced the HER2/neu presentation significantly, as compared to the corresponding minimal peptides:

HER2/neu$_{369-377}$ - DERIVED FUSION PEPTIDES

MINIMAL PEPTIDE	FUSION PEPTIDE	t RATIO	P VALUE	STATISTICALLY SIGNIFICANT (P<0.05)
HER2/neu$_{369-377}$	ES-HER$_{369-377}$	2.842	0.0295	YES
HER2/neu$_{369-377}$	IS-HER$_{369-377}$	3.215	0.0182	YES
HER2/neu$_{369-377}$	HER$_{369-377}$-IN-ES	3.267	0.0171	YES
HER2/neu$_{369-377}$	HER$_{369-377}$-IN-AF	3.394	0.0146	YES

HER2/neu$_{654-662}$ - DERIVED FUSION PEPTIDES

MINIMAL PEPTIDE	FUSION PEPTIDE	t RATIO	P VALUE	STATISTICALLY SIGNIFICANT (P<0.05)
HER2/neu$_{654-662}$	ES-HER$_{654-662}$	4.755	0.0031	YES
HER2/neu$_{654-662}$	IS-HER$_{654-662}$	4.602	0.0037	YES
HER2/neu$_{654-662}$	HER$_{654-662}$-IN-ES	2.931	0.0262	YES
HER2/neu$_{654-662}$	HER$_{654-662}$-IN-AF	3.240	0.0177	YES

LOADING OF DENDRITIC CELLS

All of the fusion peptides loaded on dendritic cells enhanced the HER2/neu presentation significantly, as compared to the corresponding minimal peptides:

MINIMAL PEPTIDE	FUSION PEPTIDE	t RATIO	P VALUE	STATISTICALLY SIGNIFICANT (P<0.05)
HER2/neu$_{369-377}$	HER$_{369-377}$-IN-AF	3.365	0.0151	YES
HER2/neu$_{654-662}$	HER$_{654-662}$-IN-AF	3.133	0.0202	YES

In order to further assess the superiority of fusion peptides over minimal peptides, we compared the duration (days) of antigen presentation yielding a value of lysis greater than 25%. The effect of loading versus duration of antigen presentation was determined for each fusion peptide and compared with that obtained with the corresponding minimal peptide by using a **paired two-tailed *t* test** (GraphPad Prism Software). We found that the fusion peptides ES-HER_{48-56}, ES-$HER_{369-377}$, ES-$HER_{654-662}$, IS-HER_{48-56}, IS-$HER_{369-377}$, and IS-$HER_{654-662}$, composed of signal sequences attached to the amino-terminus of the minimal peptides enhanced significantly the duration of antigen presentation. In contrast, the remaining fusion peptides of this group, ES-$HER_{789-797}$, and IS-$HER_{789-797}$ were not effective. Several fusion peptides of the second group, composed of minimal peptides incorporated into synthetic signal sequences, also prolonged significantly the HER2/neu antigen presentation: $HER_{369-377}$-IN-ES, $HER_{369-377}$-IN-AF, $HER_{654-662}$-IN-ES, and $HER_{654-662}$-IN-AF. In contrast, the fusion peptides HER_{48-56}-IN-ES, HER_{48-56}-IN-AF, $HER_{789-797}$-IN-ES, and $HER_{789-797}$-IN-AF were not able to enhance and prolong the presentation of the minimal peptides.

KEY RESEARCH ACCOMPLISHMENTS

INDUCTION OF CYTOTOXIC T LYMPHOCYTES FOR IMMUNOTHERAPY OF BREAST CANCER (DAMD17-00-1-0184)

- Twenty-four fusion peptides composed of natural or artificial signal sequences and HER2/neu-derived minimal peptides were constructed
- Four CTL lines recognizing the HER2/neu-derived peptides HER2/neu$_{48-56}$, HER2/neu$_{369-377}$, HER2/neu$_{654-662}$, and HER2/neu$_{789-797}$ were generated by *in vitro* immunization of peripheral blood mononuclear cells
- Antigen processing-deficient T2 cells loaded with most of the constructs composed of signal sequence at the amino-terminus of HER2/neu peptides were recognized by CTL up to eight days after loading
- Replacing of the hydrophobic region of several signal sequences with the hydrophobic HER2/neu peptides resulted in a very efficient presentation of these epitopes
- The results of the IFN-γ release assays confirmed that the most efficient constructs in facilitating the HER2/neu peptide presentation are the constructs of the type HER-IN-AF
- We demonstrated that the most effective signal sequences, already selected in the experiments with the TAP-deficient T2 cells, could also improve HER2/neu antigen presentation in human breast cancer cells
- The results of the IFN-γ release assays confirmed that the most efficient constructs in facilitating the HER2/neu peptide presentation in breast cancer cells are the constructs of the type HER-IN-AF
- Using three modern approaches for studying the mechanisms of antigen presentation, we confirmed that the signal sequence-loaded antigenic peptides were introduced into the cytosol of the cells, resulting in a prolonged and more efficient antigen presentation
- Using a novel system for screening different fusion peptides for TAP-**in**dependent translocation of peptide antigens through the ER-membrane, we confirmed that the signal sequence approach is very effective in improving antigen presentation, even in tumor cells with deficiency of antigen processing/presentation.
- We found that the most effective signal sequences could also improve and prolong the presentation of HER2/neu peptides in human dendritic cells (DC)
- Importantly, we demonstrated that the prolonged presentation of the HER2/neu peptides by the dendritic cells actually improves the generation of HER2/neu-specific CTL *in vitro*
- We confirmed by ELISPOT that the peptide-loaded DC can improve the priming of CTL by increasing the number of antigen-specific, IFNγ-producing cells
- All results were analyzed for statistical significance using a paired two-tailed *t* test (GraphPad Prism Software).

REPORTABLE OUTCOMES

INDUCTION OF CYTOTOXIC T LYMPHOCYTES FOR IMMUNOTHERAPY OF BREAST CANCER (DAMD17-00-1-0184)

1. **Manuscript:** BORIS R. MINEV, FANG GUO, IVELINA GUEORGUIEVA and HANS E. KAISER.: Vaccines for Immunotherapy of Breast Cancer and Prostate Cancer: New Developments and Comparative Aspects. *In Vivo* 15 (5), 2002 (In Press)

2. **Abstract:** BORIS R. MINEV, JASON HIPP and JENNIFER HIPP.: Development of Synthetic Vaccines for Immunotherapy of Breast Cancer. *Era of Hope Meeting,* Orlando, Florida, September 25-28, 2002

3. **United States Provisional Patent Application:** BORIS R. MINEV: Enhancing Class I Antigen Presentation With Synthetic Sequences. *UCSD Technology Transfer & IP Services,* July 11, 2003

CONCLUSIONS

INDUCTION OF CYTOTOXIC T LYMPHOCYTES FOR IMMUNOTHERAPY OF BREAST CANCER (DAMD17-00-1-0184)

Based on our observations in this study we conclude that the position of the synthetic signal sequence relative to the HER2/neu-derived class I restricted epitopes is critical to its ability to facilitate antigen presentation. NH_2-terminal localization of the signal sequence is advantageous for antigen presentation probably because the signal peptidase requires this configuration to cleave the minimal peptide in the ER. We have explored the post-translational translocation of fusion peptides through the ER-membrane. For most proteins, translocation across mammary ER membrane has a strict requirement for co-translational delivery of the nascent chain (8). However, several studies in eukaryotic cells identified a variety of proteins that can also be translocated post-translationally (9) (10) (11). These studies confirm our finding that both co-translational and post-translational pathways coexist in eukaryotes.

We do not know the exact mechanism of translocation of our constructs through the ER membrane. However, the lack of recognition of T2 cells loaded with the minimal HER2/neu peptides 3 days after loading suggests that minimal peptide alone was not translocated into the ER, and therefore, was not presented on the surface of the loaded T2 cells. The signal sequence at the amino-terminus may enhance the translocation of the minimal peptide due to the higher hydrophobicity or higher resistance of fusion peptides to proteolytic enzymes. However, loading of the T2 cells with HER-ES and HER-IS, whose hydrophobicity was determined to be nearly identical to that of ES-HER and IS-HER, was not efficient.

In our experiments we exploited the hydrophobic nature of the HER2/neu peptides to demonstrate that they could be presented on the surface of T2 cells when incorporated into a signal sequence. In agreement with our finding, Gueguen et al. showed with recombinant vaccinia vectors that a peptide derived from a signal sequence cleaved in the ER can provide an epitope for HLA-A2 restricted T cell recognition in the TAP-

deficient T2 cells (12). The ER is a proteolytically active environment, capable of generating Class I binding peptides (13). On the other hand, two studies found that functional TAP was required for efficient presentation of Class I restricted epitopes from influenza (14) and lymphocytic choriomeningitis virus (15) incorporated into signal sequences. This suggests that in some cases cytosolic degradation rather than ER proteolysis is responsible for generating the Class I binding epitopes. It seems likely that different fusion proteins fold differently in the cytosol and some of them may assume a translocation-unfavorable state and are degraded in the cytosol.

In this study we have shown that synthetic insertion signal sequences can enhance and prolong presentation of HER2/neu in the TAP-deficient T2 cells, breast cancer cells, and human dendritic cells. This mechanism may be especially important in cancers that fail to utilize the classical Class I pathway (16) (17).

In another set of experiments we studied the mechanisms involved in the enhancement of antigen presentation by the fusion peptides. By performing acid stripping, pronase treatment, and Brefeldin treatment of the peptide-loaded tumor cells we proved that the effective presentation of the loaded peptide constructs is a result of their efficient loading into the cytosol and not simple binding to the surface HLA molecules. We also studied the role of TAP in class I presentation in human cancer cells, and tested the efficiency of different signal peptides in cancer cells with different levels of TAP expression. Using a novel system for screening different fusion peptides for TAP-**in**dependent translocation of peptide antigens through the ER-membrane, we confirmed that the signal sequence approach is very effective in improving antigen presentation, even in tumor cells with deficiency of antigen processing/presentation.

Peptide-loaded dendritic cells (DC) are currently being used in clinical protocols for cancer immunotherapy (18) (19). It has not been established whether DC can be internally loaded with peptide constructs in order to improve and prolong the presentation of the loaded peptides. We demonstrated: (i) that human DC can be loaded efficiently with the fusion peptides; and (ii) that we can improve significantly the efficiency of the *in vitro* immunization of human CTL by using fusion peptide-loaded DC. These experiments provided important insights on the possibility to improve the efficiency as well as to shorten the time needed for the CTL induction.

Fusing protein signal sequences to HLA Class I-restricted minimal peptides may be helpful for the development of synthetic vaccines against neoplastic and viral diseases. Immunizing with minimal determinant constructs may avoid the possible oncogenic effect of full-length proteins containing ras, p53 or other potential oncogenes. In addition, *in vivo* or *in vitro* immunization with peptide antigens "packaged" in dendritic cells or other antigen-presenting cells opens an exciting opportunity for eliciting powerful CTL-responses. The general problem limiting the use of peptide vaccines in humans is that T cells from individuals expressing different MHC molecules recognize different peptides from tumor or viral antigens in the context of self MHC. However, the use of synthetic epitopes from tumor-associated antigens presented by commonly expressed MHC molecules makes the use of such vaccines feasible. Thus, our findings may be applicable to the development of peptide vaccines for *in vivo* and *in vitro* specific immunization.

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INDUCTION OF CYTOTOXIC T LYMPHOCYTES FOR IMMUNOTHERAPY OF BREAST CANCER (DAMD17-00-1-0184)

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Table 1.

HER2/neu-derived HLA-A2 restricted peptides

PEPTIDES	SEQUENCE	LOCATION	REFERENCE
HER2/neu$_{48-56}$	HLYQGCQVV	EXTRACELLULAR	Disis, M.(Cancer Res. 54:1071-6, 1994)
HER2/neu$_{369-377}$	KIFGSLAFL	EXTRACELLULAR	Fisk, B.(J.Exp.Med. 181:2109-17, 1995)
HER2/neu$_{654-662}$	IISAVVGIL	TRANSMEMBRANE	Peoples,G.(P.N.A.S 92:432-6, 1995)
HER2/neu$_{789-797}$	CLTSTVQLV	INTRACELLULAR	Disis, M.(Cancer Res. 54:1071-6, 1994)

Table 2.

Synthetic peptide constructs with HER2/neu$_{48-56}$

DESIGNATION	PEPTIDE SEQUENCE
1. HER - HER2/neu$_{48-56}$	**HLYQGCQVV**
2. ES-HER$_{48-56}$	M R Y M I L G L L A L A A V C S A **HLYQGCQVV**
3. HER$_{48-56}$-ES	**HLYQGCQVV** M R Y M I L G L L A L A A V C S A
4. IS-HER$_{48-56}$	M T N K C L L Q I A L L L C F S T T A L S **HLYQGCQVV**
5. HER$_{48-56}$-IS	**HLYQGCQVV** M T N K C L L Q I A L L L C F S T T A L S
6. HER$_{48-56}$-IN-ES	M R **HLYQGCQVV** A A V C S A
7. HER$_{48-56}$-IN-AF	M A **HLYQGCQVV** A A A A A G

Synthetic peptide constructs:

1. Peptide antigen HER2/neu$_{48-56}$
2. Adenoviral signal sequence ES attached to the amino-terminus of HER2/neu$_{48-56}$
3. Adenoviral signal sequence ES attached to the carboxy-terminus of HER2/neu$_{48-56}$
4. Interferon signal sequence IS attached to the amino-terminus of HER2/neu$_{48-56}$
5. Interferon signal sequence IS attached to the carboxy-terminus of HER2/neu$_{48-56}$
6. Peptide antigen HER2/neu$_{48-56}$ replacing the hydrophobic portion of ES
7. Peptide antigen HER2/neu$_{48-56}$ incorporated into an artificial signal sequence - AF

Table 3.

Synthetic peptide constructs with HER2/neu$_{369-377}$

DESIGNATION	PEPTIDE SEQUENCE
1. HER - HER2/neu$_{369-377}$	**KIFGSLAFL**
2. ES-HER$_{369-377}$	M R Y M I L G L L A L A A V C S A **KIFGSLAFL**
3. HER$_{369-377}$-ES	**KIFGSLAFL** M R Y M I L G L L A L A A V C S A
4. IS-HER$_{369-377}$	M T N K C L L Q I A L L L C F S T T A L S **KIFGSLAFL**
5. HER$_{369-377}$-IS	**KIFGSLAFL** M T N K C L L Q I A L L L C F S T T A L S
6. HER$_{369-377}$-IN-ES	M R **KIFGSLAFL** A A V C S A
7. HER$_{369-377}$-IN-AF	M A **KIFGSLAFL** A A A A A G

Table 4.

Synthetic peptide constructs with HER2/neu$_{654-662}$

DESIGNATION	PEPTIDE SEQUENCE
1. HER - HER2/neu$_{654-662}$	**IISAVVGIL**
2. ES-HER$_{654-662}$	M R Y M I L G L L A L A A V C S A **IISAVVGIL**
3. HER$_{654-662}$-ES	**IISAVVGIL** M R Y M I L G L L A L A A V C S A
4. IS-HER$_{654-662}$	M T N K C L L Q I A L L L C F S T T A L S **IISAVVGIL**
5. HER$_{654-662}$-IS	**IISAVVGIL** M T N K C L L Q I A L L L C F S T T A L S
6. HER$_{654-662}$-IN-ES	M R **IISAVVGIL** A A V C S A
7. HER$_{654-662}$-IN-AF	M A **IISAVVGIL** A A A A A G

Table 5.

Synthetic peptide constructs with HER2/neu$_{789-797}$

DESIGNATION	PEPTIDE SEQUENCE
1. HER - HER2/neu$_{789-797}$	**CLTSTVQLV**
2. ES-HER$_{789-797}$	M R Y M I L G L L A L A A V C S A **CLTSTVQLV**
3. HER$_{789-797}$-ES	**CLTSTVQLV** M R Y M I L G L L A L A A V C S A
4. IS-HER$_{789-797}$	M T N K C L L Q I A L L L C F S T T A L S **CLTSTVQLV**
5. HER$_{789-797}$-IS	**CLTSTVQLV** M T N K C L L Q I A L L L C F S T T A L S
6. HER$_{789-797}$-IN-ES	M R **CLTSTVQLV** A A V C S A
7. HER$_{789-797}$-IN-AF	M A **CLTSTVQLV** A A A A A G

Table 6.

^{51}Cr-release assay using T2 cells pulsed with HER2/neu-derived peptides as targets for CTL

E:T ratio	50:1	25:1	12:1	6:1	3:1	1.5:1
T2	1	2	1	0	1	0
T2 pulsed with HER2/neu$_{48-56}$	88	53	41	33	19	8
T2 pulsed with HER2/neu$_{369-377}$	94	66	57	42	23	12
T2 pulsed with HER2/neu$_{654-662}$	91	71	59	38	26	13
T2 pulsed with HER2/neu$_{789-797}$	83	62	52	31	22	9

Table 7.
Release of IFNγ by CTL after incubation with non-loaded or peptide-loaded T2 cells

A. Day 1 after peptide loading

CTL elicited with:	Stimulators in ELISA assays: T2 cells loaded with: [a]							
	--	HER	ES-HER	HER-ES	IS-HER	HER-IS	HER-IN-ES	HER-IN-AF
HER2/neu$_{48-56}$	210 [b]	2280	2894	418	3268	288	3368	3488
HER2/neu$_{369-377}$	186	3120	3368	172	2120	212	2227	3288
HER2/neu$_{654-662}$	121	2827	2667	144	2590	111	2929	3321
HER2/neu$_{789-797}$	234	2924	1824	58	1717	69	246	296

B. Day 3 after peptide loading

CTL elicited with:	Stimulators in ELISA assays: T2 cells loaded with: [a]							
	--	HER	ES-HER	HER-ES	IS-HER	HER-IS	HER-IN-ES	HER-IN-AF
HER2/neu$_{48-56}$	129 [b]	488	1876	218	1264	148	1349	1229
HER2/neu$_{369-377}$	143	127	2377	142	1818	112	2029	2401
HER2/neu$_{654-662}$	111	429	2518	124	1990	99	2773	2981
HER2/neu$_{789-797}$	215	317	526	78	315	78	312	327

[a]CTL were coincubated with stimulator cells (non-loaded or peptide-loaded T2 cells) for 20 h. The concentration of IFNγ in coculture supernatants was then determined by ELISA.

[b]IFNγ (pg/ml) - mean numbers of IFNγ release in triplicate wells with 10^5 CTL/well

Table 7.
Release of IFNγ by CTL after incubation with non-loaded or peptide-loaded T2 cells

C. Day 5 after peptide loading

CTL elicited with:	Stimulators in ELISA assays: T2 cells loaded with: [a]							
	–	HER	ES-HER	HER-ES	IS-HER	HER-IS	HER-IN-ES	HER-IN-AF
HER2/neu$_{48-56}$	181 [b]	134	953	99	943	155	988	1010
HER2/neu$_{369-377}$	111	211	1073	121	1323	117	1663	1773
HER2/neu$_{654-662}$	97	121	1245	137	1557	121	1699	1892
HER2/neu$_{789-797}$	136	116	168	69	125	87	121	178

D. Day 8 after peptide loading

CTL elicited with:	Stimulators in ELISA assays: T2 cells loaded with: [a]							
	–	HER	ES-HER	HER-ES	IS-HER	HER-IS	HER-IN-ES	HER-IN-AF
HER2/neu$_{48-56}$	116 [b]	177	589	101	614	121	228	718
HER2/neu$_{369-377}$	93	89	592	118	545	103	690	878
HER2/neu$_{654-662}$	115	167	581	83	615	76	671	881
HER2/neu$_{789-797}$	88	91	110	98	104	59	107	117

[a]CTL were coincubated with stimulator cells (non-loaded or peptide-loaded T2 cells) for 20 h. The concentration of IFNγ in coculture supernatants was then determined by ELISA.

[b]IFNγ (pg/ml) - mean numbers of IFNγ release in triplicate wells with 10^5 CTL/well

Table 8.

Lack of recognition of breast cancer cell line MDA-MB-231 by CTL reactive against HER2/neu$_{369-377}$ and HER2/neu$_{654-662}$

			Percent ^{51}Cr Released From[a]:		
Effectors	**E:T**	**T2**	**T2-pulsed[b]**	**MCF7**	**MDA-MB-231**
CTL$_{369-377}$	40:1	2	94	68	2
	20:1	1	73	33	1
CTL$_{654-662}$	40:1	1	71	58	2
	20:1	2	57	31	0

a) Cytotoxicity was evaluated in a 6-hour ^{51}Cr-release assay.
b) T2 cells were pulsed with HER2/neu$_{369-377}$, or HER2/neu$_{654-662}$ at 1 μg/ml for 2 hours at 37°C, labeled with ^{51}Cr and used as targets.

Table 9.

Release of IFNγ by CTL after incubation with non-loaded or peptide-loaded breast cancer cells MDA-MB-231

A. Day 1 after peptide loading

CTL elicited with:	Stimulators in ELISA assays: MDA-MB-231 cells loaded with: [a]							
	--	HER	ES-HER	HER-ES	IS-HER	HER-IS	HER-IN-ES	HER-IN-AF
HER2/neu$_{369-377}$	177[b]	2820	3688	182	2126	224	2627	3381
HER2/neu$_{654-662}$	153	2826	2767	188	2678	144	2727	3321

B. Day 3 after peptide loading

CTL elicited with:	Stimulators in ELISA assays: MDA-MB-231 cells loaded with: [a]							
	--	HER	ES-HER	HER-ES	IS-HER	HER-IS	HER-IN-ES	HER-IN-AF
HER2/neu$_{369-377}$	138[b]	141	2417	187	1777	131	2187	2347
HER2/neu$_{654-662}$	121	438	2622	138	1974	102	2666	2994

C. Day 5 after peptide loading

CTL elicited with:	Stimulators in ELISA assays: MDA-MB-231 cells loaded with: [a]							
	--	HER	ES-HER	HER-ES	IS-HER	HER-IS	HER-IN-ES	HER-IN-AF
HER2/neu$_{369-377}$	122[b]	274	1278	137	1444	128	1778	1897
HER2/neu$_{654-662}$	102	131	1445	135	1604	132	1708	1933

D. Day 8 after peptide loading

CTL elicited with:	Stimulators in ELISA assays: MDA-MB-231 cells loaded with: [a]							
	--	HER	ES-HER	HER-ES	IS-HER	HER-IS	HER-IN-ES	HER-IN-AF
HER2/neu$_{369-377}$	102[b]	99	577	122	587	113	687	889
HER2/neu$_{654-662}$	125	147	578	93	628	86	667	899

[a]CTL were coincubated with stimulator cells (non-loaded or peptide-loaded MDA-MB-231 cells) for 20 h. The concentration of IFNg in coculture supernatants was then determined by ELISA.

[b]IFNg (pg/ml) - mean numbers of IFNg release in triplicate wells with 10^5 CTL/well

Table 10.

Mechanisms of peptide loading: Recognition of breast cancer cells MDA-MB-231 by CTL reactive against HER2/neu$_{369-377}$ and HER2/neu$_{654-662}$

		Percent ^{51}Cr Released From MDA-MB-231 cells treated with[a]:			
Effectors	**E:T**	**acid**	**pronase**	**brefeldin**	**non-treated**
CTL$_{369-377}$	40:1	62	59	18	62
	20:1	41	33	7	39
CTL$_{654-662}$	40:1	61	61	13	58
	20:1	28	37	6	30

a) Cytotoxicity was evaluated in a 6-hour ^{51}Cr-release assay 3 days after peptide loading

Table 11.

Mechanisms of peptide loading: Recognition of ICP47-transfected cells MCF7 by CTL reactive against HER2/neu$_{369-377}$ and HER2/neu$_{654-662}$

		Percent ^{51}Cr Released From MCF7 cells loaded with[a]:					
Effectors	**E:T**	**MCF7**	**MCF7-ICP47**	**ES-HER**	**IS-HER**	**HER-IN-ES**	**HER-IN-AF**
CTL$_{369-377}$	40:1	62	3	1	2	4	58
	20:1	29	3	2	3	3	24
CTL$_{654-662}$	40:1	74	4	3	5	2	66
	20:1	31	2	2	3	2	22

a) Cytotoxicity was evaluated in a 6-hour ^{51}Cr-release assay 3 days after peptide loading

Table 12.

Enumeration of IFNγ–releasing cells among CTL elicited after two cycles of stimulation with peptide-loaded dendritic cells.

A. DC loaded with $HER_{369-377}$ or $HER_{369-377}$-IN-AF

	Stimulator in ELISPOT Assay[a]:	
CTL elicited with:	**T2**	**T2($HER_{369-377}$)**
DC	20[b]	22
DC loaded with $HER_{369-377}$	21	31
DC loaded with $HER_{369-377}$-IN-AF	22	108

a) CTL were tested for IFN-γ release after 20 hours of incubation with non-pulsed or $HER_{369-377}$-pulsed T2 cells.

b) Mean numbers of spots in triplicate wells with 10^5 CTL/well

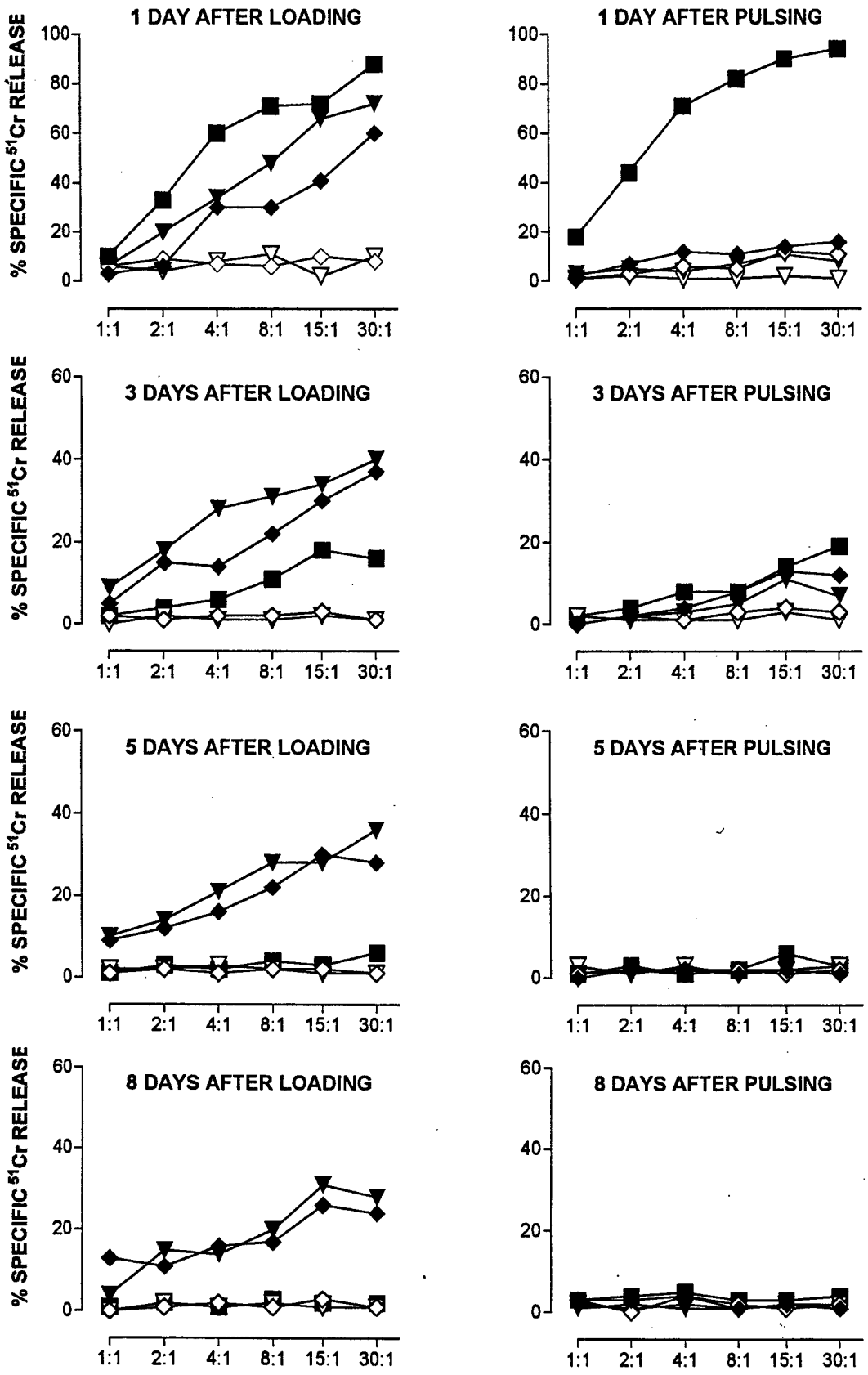
B. DC loaded with $HER_{654-662}$ or $HER_{654-662}$-IN-AF

	Stimulator in ELISPOT Assay[a]:	
CTL elicited with:	**T2**	**T2($HER_{654-662}$)**
DC	17[b]	19
DC loaded with $HER_{654-662}$	18	32
DC loaded with $HER_{654-662}$-IN-AF	24	98

a) CTL were tested for IFN-γ release after 20 hours of incubation with non-pulsed or $HER_{654-662}$-pulsed T2 cells.

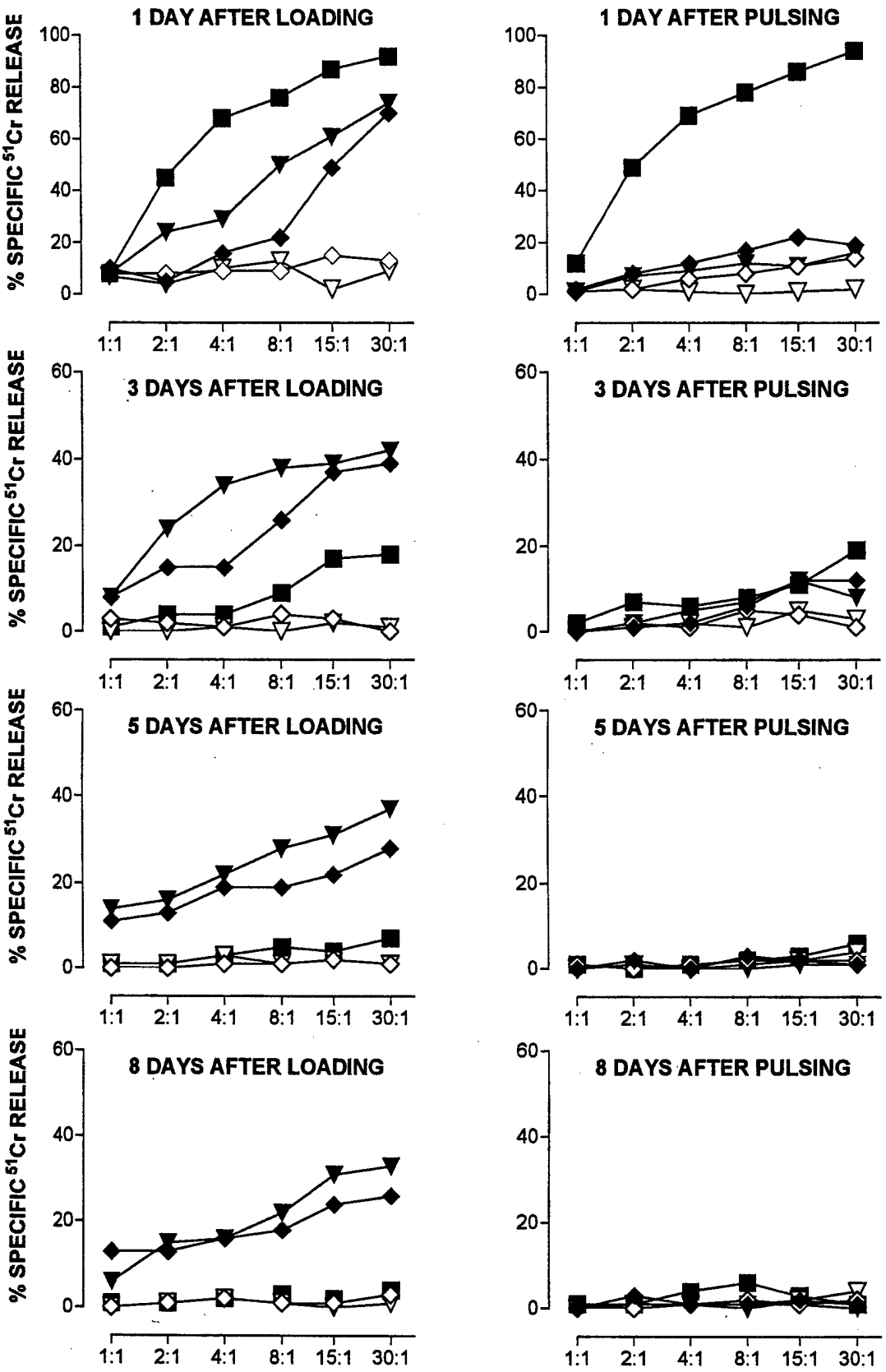
b) Mean numbers of spots in triplicate wells with 10^5 CTL/well

Figure 1.



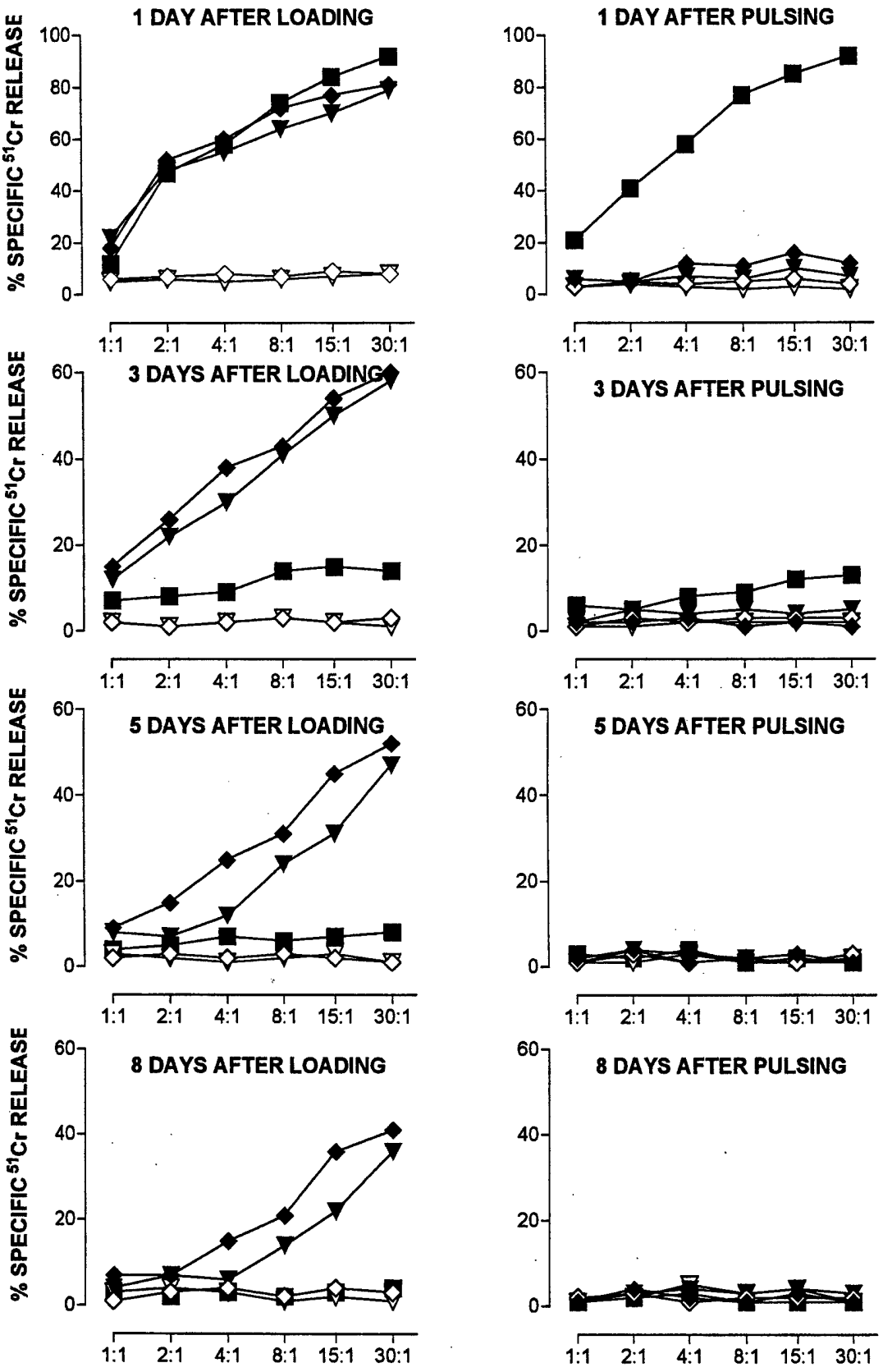
Loading/pulsing of T2 cells with peptide constructs composed of synthetic signal sequences attached to amino-terminus or to carboxy-terminus of HER2/neu$_{48\text{-}56}$. T2 cells were loaded (left column) or pulsed (right column) with ES-HER$_{48\text{-}56}$ (◆), HER$_{48\text{-}56}$-ES (◇), IS-HER$_{48\text{-}56}$ (▼), HER$_{48\text{-}56}$-IS (▽), or HER$_{48\text{-}56}$ (■). At different periods after loading, T2 cells were used as targets in ^{51}Cr-release assays for the CTL.

Figure 2.



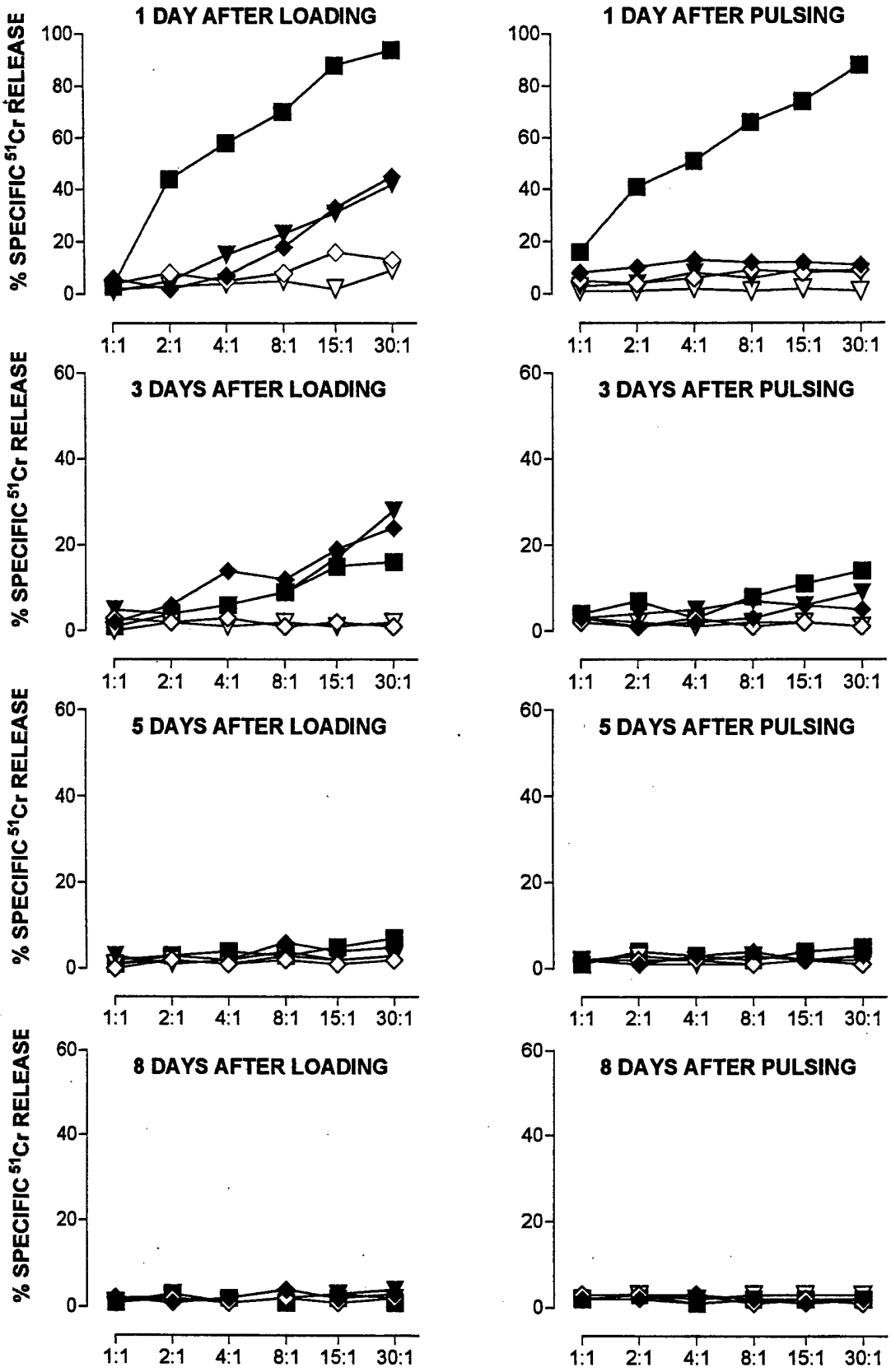
Loading/pulsing of T2 cells with peptide constructs composed of synthetic signal sequences attached to amino-terminus or to carboxy-terminus of HER2/neu$_{369\text{-}377}$. T2 cells were loaded (left column) or pulsed (right column) with ES-HER$_{369\text{-}377}$ (◆), HER$_{369\text{-}377}$-ES (◇), IS-HER$_{369\text{-}377}$ (▼), HER$_{369\text{-}377}$-IS (▽), or HER$_{369\text{-}377}$ (■). At different periods after loading, T2 cells were used as targets in ^{51}Cr-release assays for the CTL.

Figure 3.



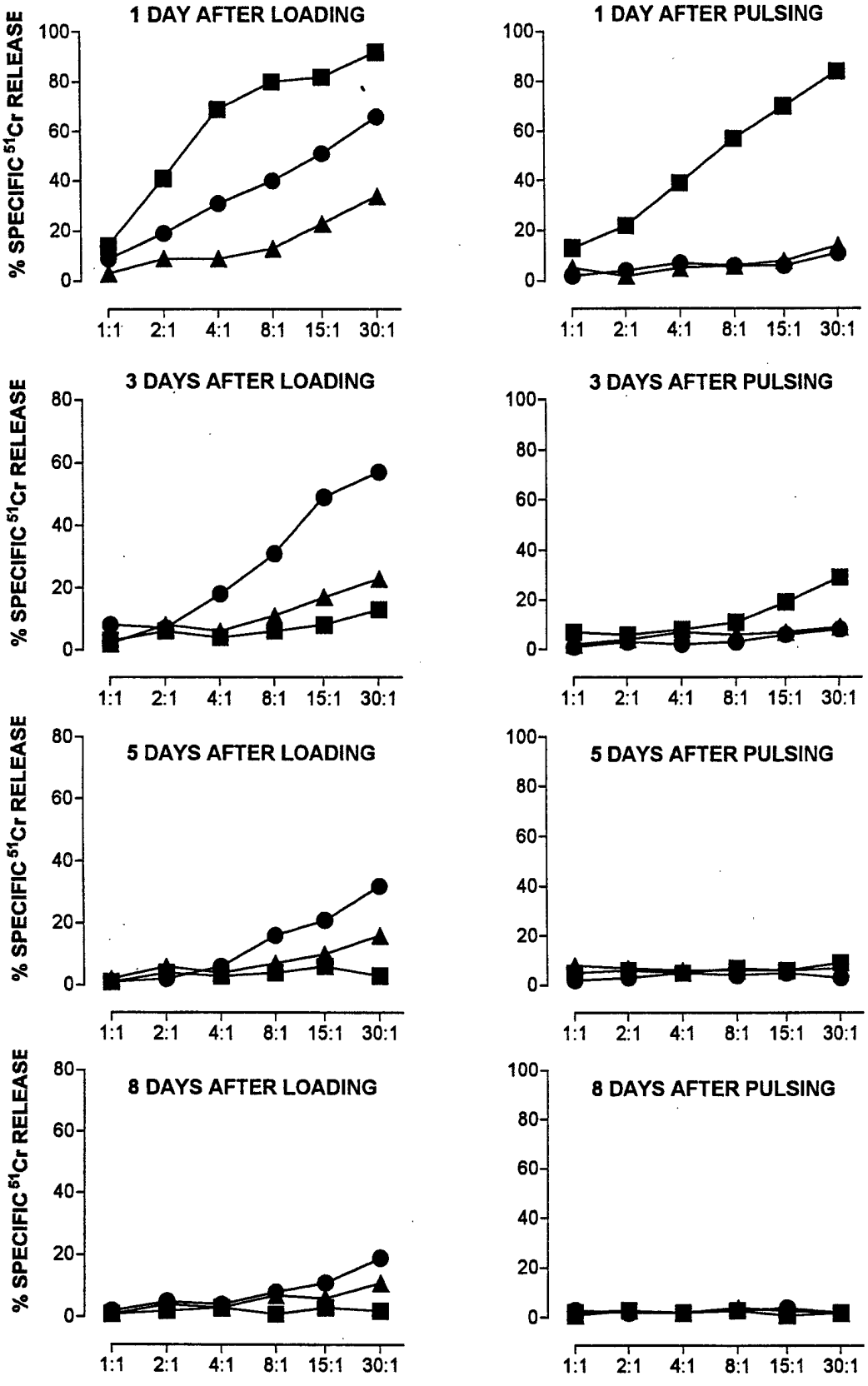
Loading/pulsing of T2 cells with peptide constructs composed of synthetic signal sequences attached to amino-terminus or to carboxy-terminus of HER2/neu$_{654-662}$. T2 cells were loaded (left column) or pulsed (right column) with ES-HER$_{654-662}$ (◆), HER$_{654-662}$-ES (◇), IS-HER$_{654-662}$ (▼), HER$_{654-662}$-IS (▽), or HER$_{654-662}$ (■). At different periods after loading, T2 cells were used as targets in ^{51}Cr-release assays for the CTL.

Figure 4.



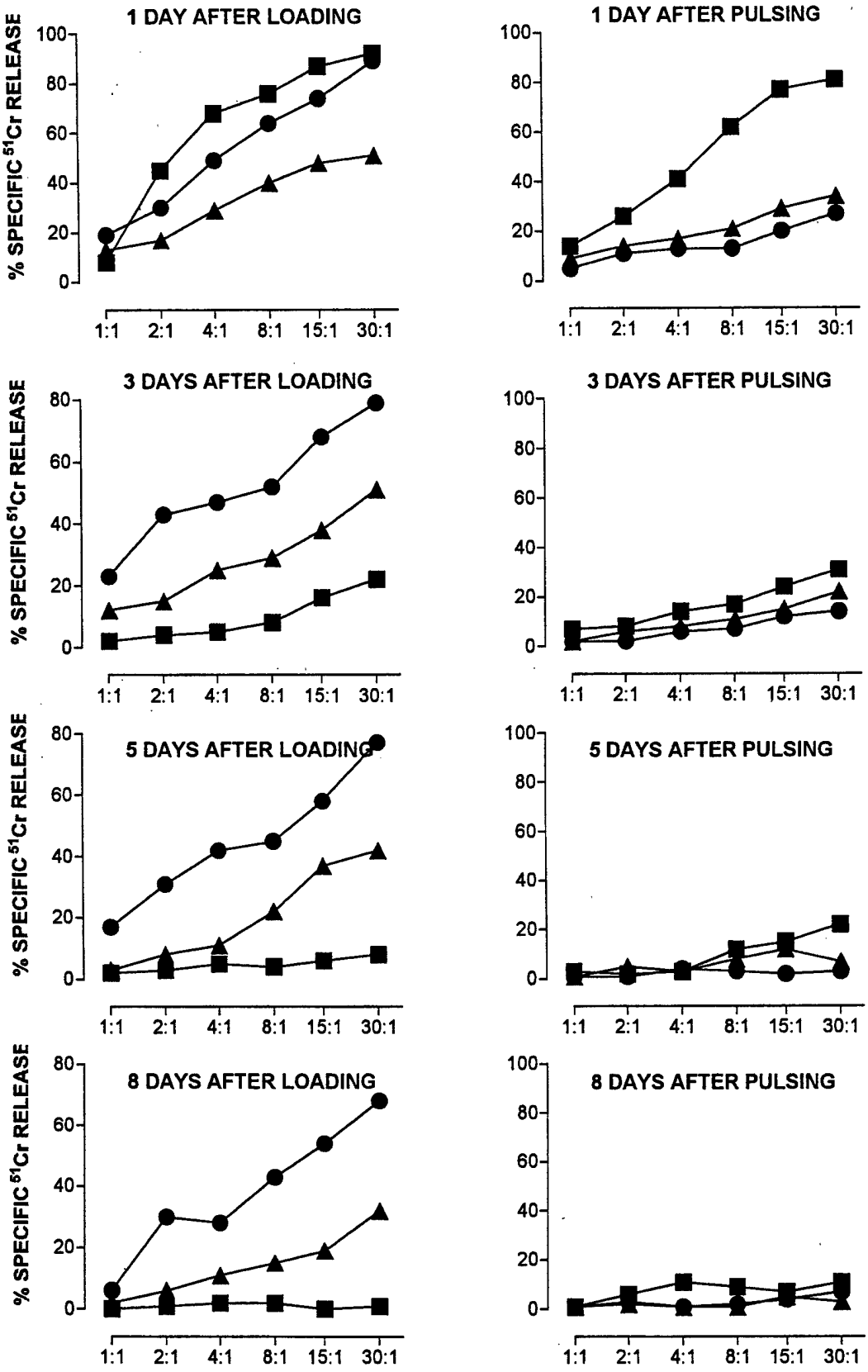
Loading/pulsing of T2 cells with peptide constructs composed of synthetic signal sequences attached to amino-terminus or to carboxy-terminus of HER2/neu$_{789\text{-}797}$. T2 cells were loaded (left column) or pulsed (right column) with ES-HER$_{789\text{-}797}$ (◆), HER$_{789\text{-}797}$-ES (◇), IS-HER$_{789\text{-}797}$ (▼), HER$_{789\text{-}797}$-IS (▽), or HER$_{789\text{-}797}$ (■). At different periods after loading, T2 cells were used as targets in ^{51}Cr-release assays for the CTL.

Figure 5.



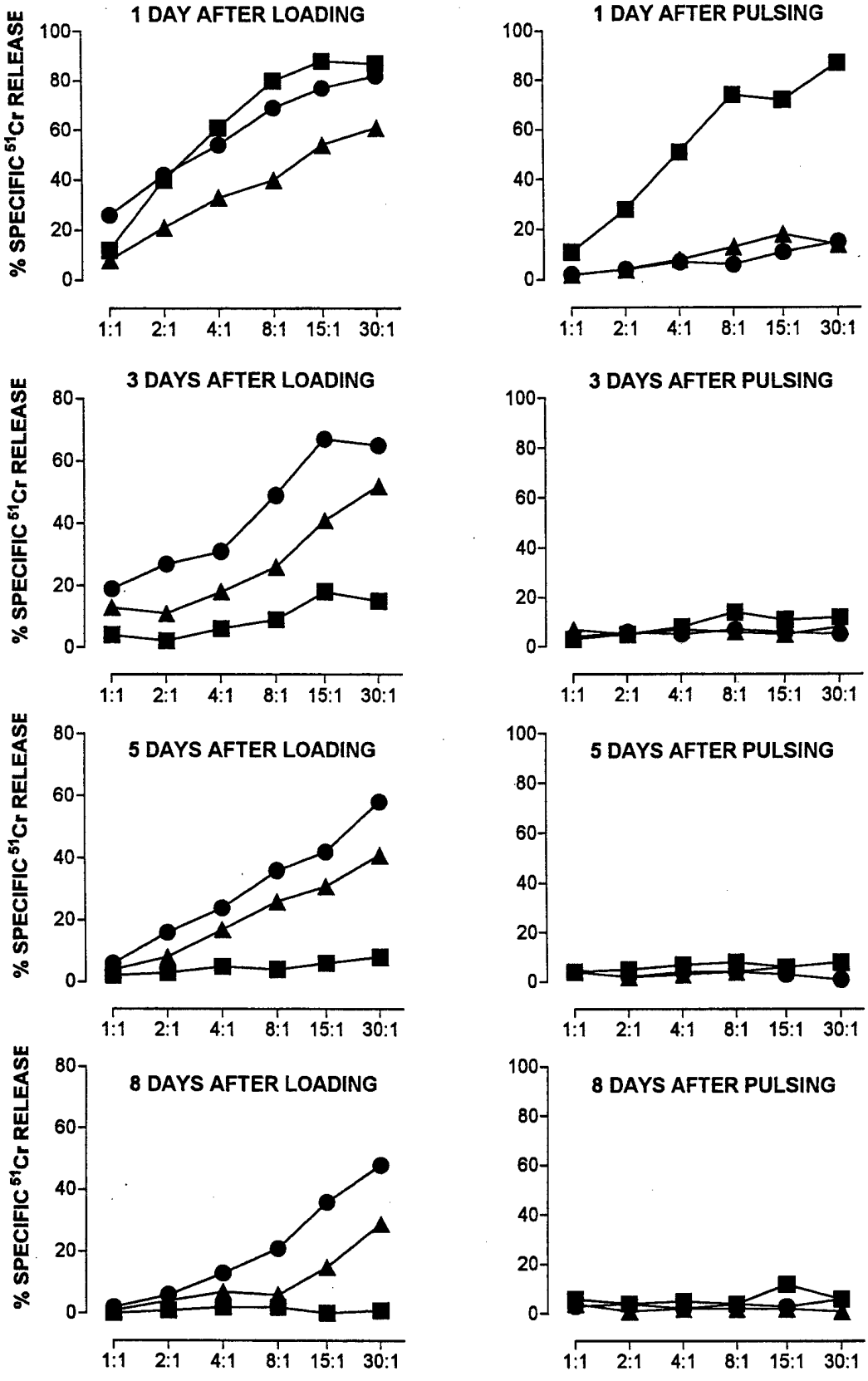
Loading/pulsing of T2 cells with peptide constructs composed of HER2/neu $_{48\text{-}56}$ incorporated into synthetic signal sequences. T2 cells were loaded (left column) or pulsed (right column) with $HER_{48\text{-}56}$-IN-AF (●), $HER_{48\text{-}56}$-IN-ES (▲), or $HER_{48\text{-}56}$ (■). At different periods after loading, T2 cells were used as targets in ^{51}Cr-release assays for the CTL.

Figure 6.



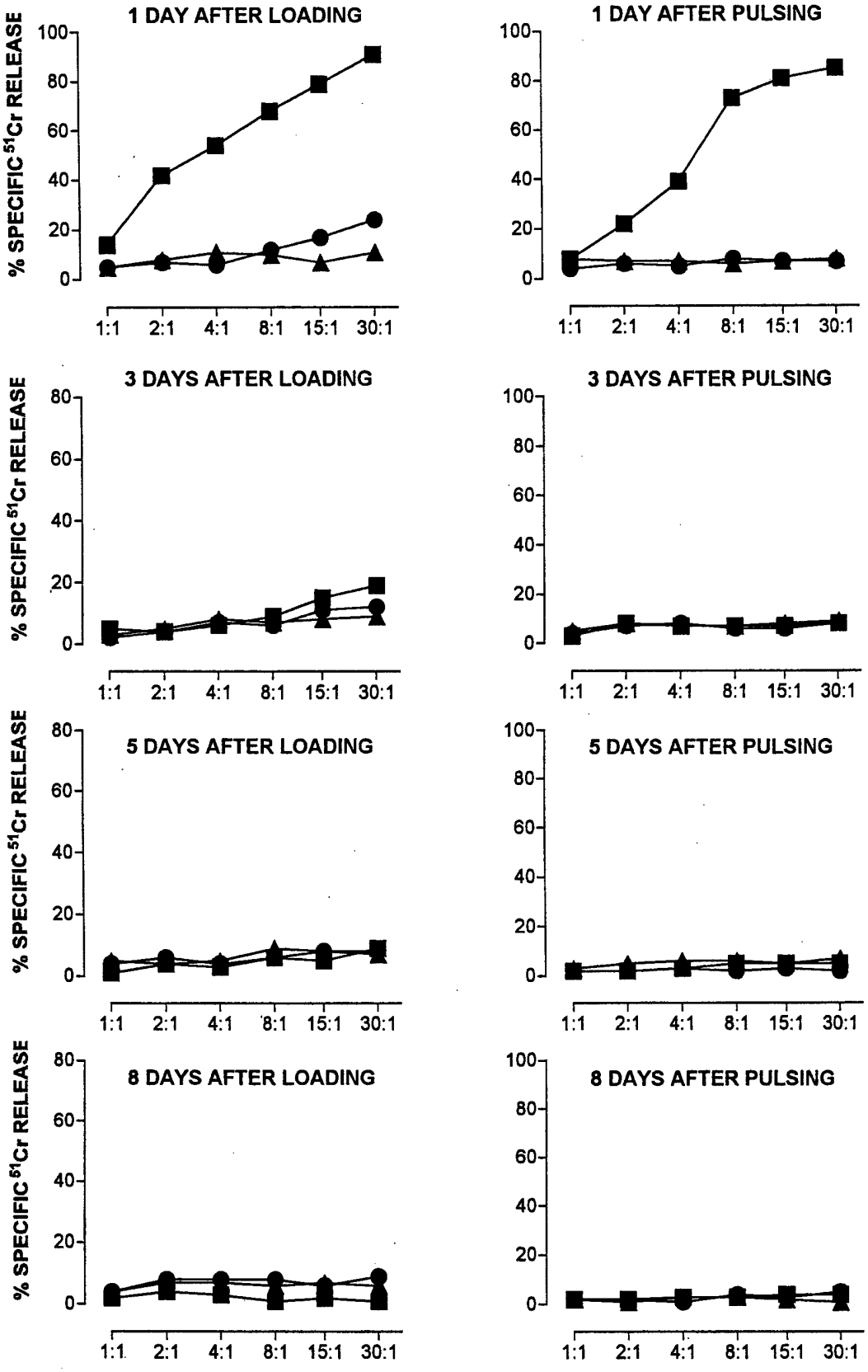
Loading/pulsing of T2 cells with peptide constructs composed of HER2/$neu_{369-377}$ incorporated into synthetic signal sequences. T2 cells were loaded (left column) or pulsed (right column) with $HER_{369-377}$-IN-AF (●), $HER_{369-377}$-IN-ES (▲), or $HER_{369-377}$ (■). At different periods after loading, T2 cells were used as targets in ^{51}Cr-release assays for the CTL.

Figure 7.



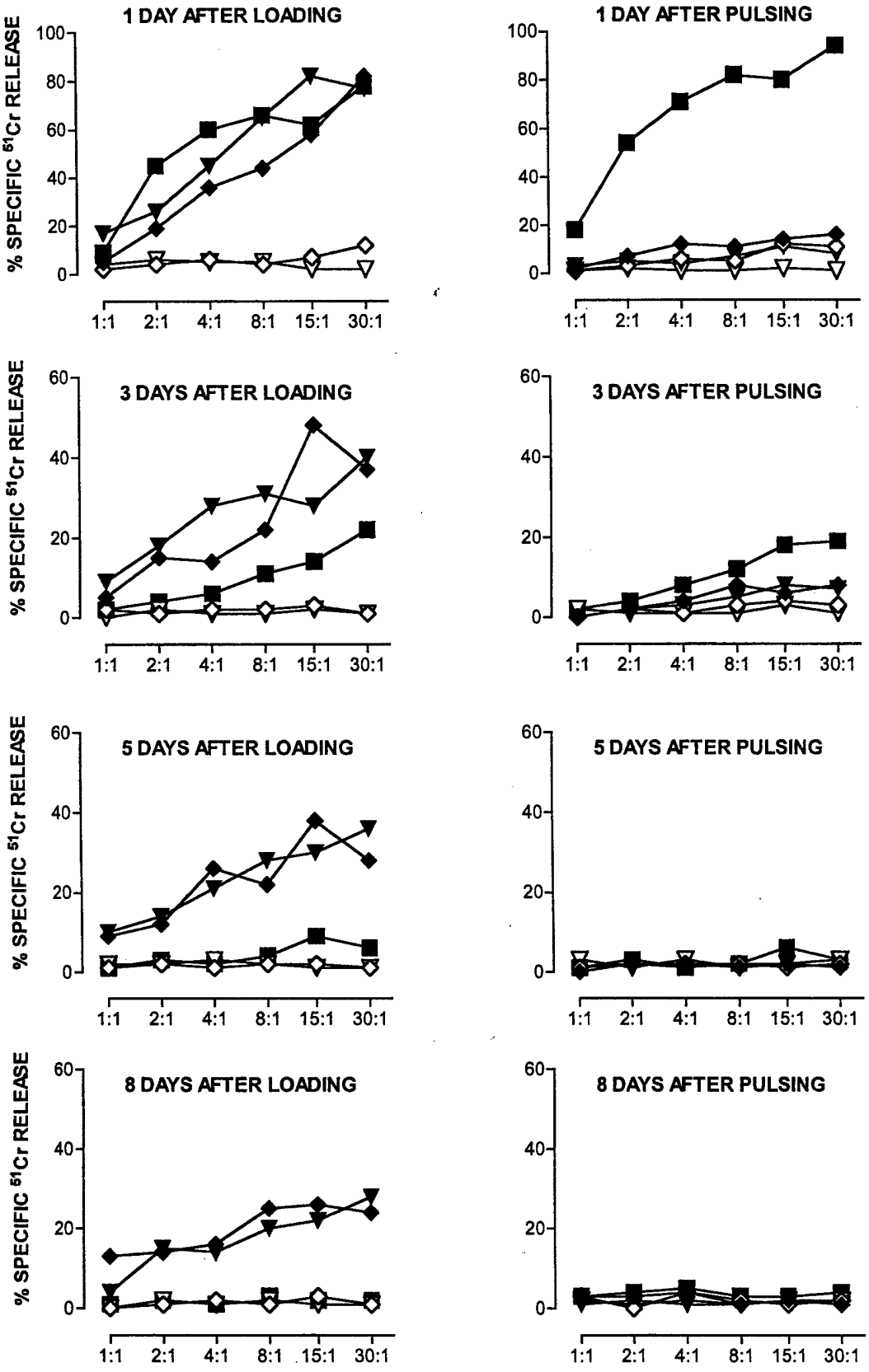
Loading/pulsing of T2 cells with peptide constructs composed of HER2/neu$_{654-662}$ incorporated into synthetic signal sequences. T2 cells were loaded (left column) or pulsed (right column) with HER$_{654-662}$-IN-AF (●), HER$_{654-662}$-IN-ES (▲), or HER$_{654-662}$ (■). At different periods after loading, T2 cells were used as targets in ^{51}Cr-release assays for the CTL.

Figure 8.



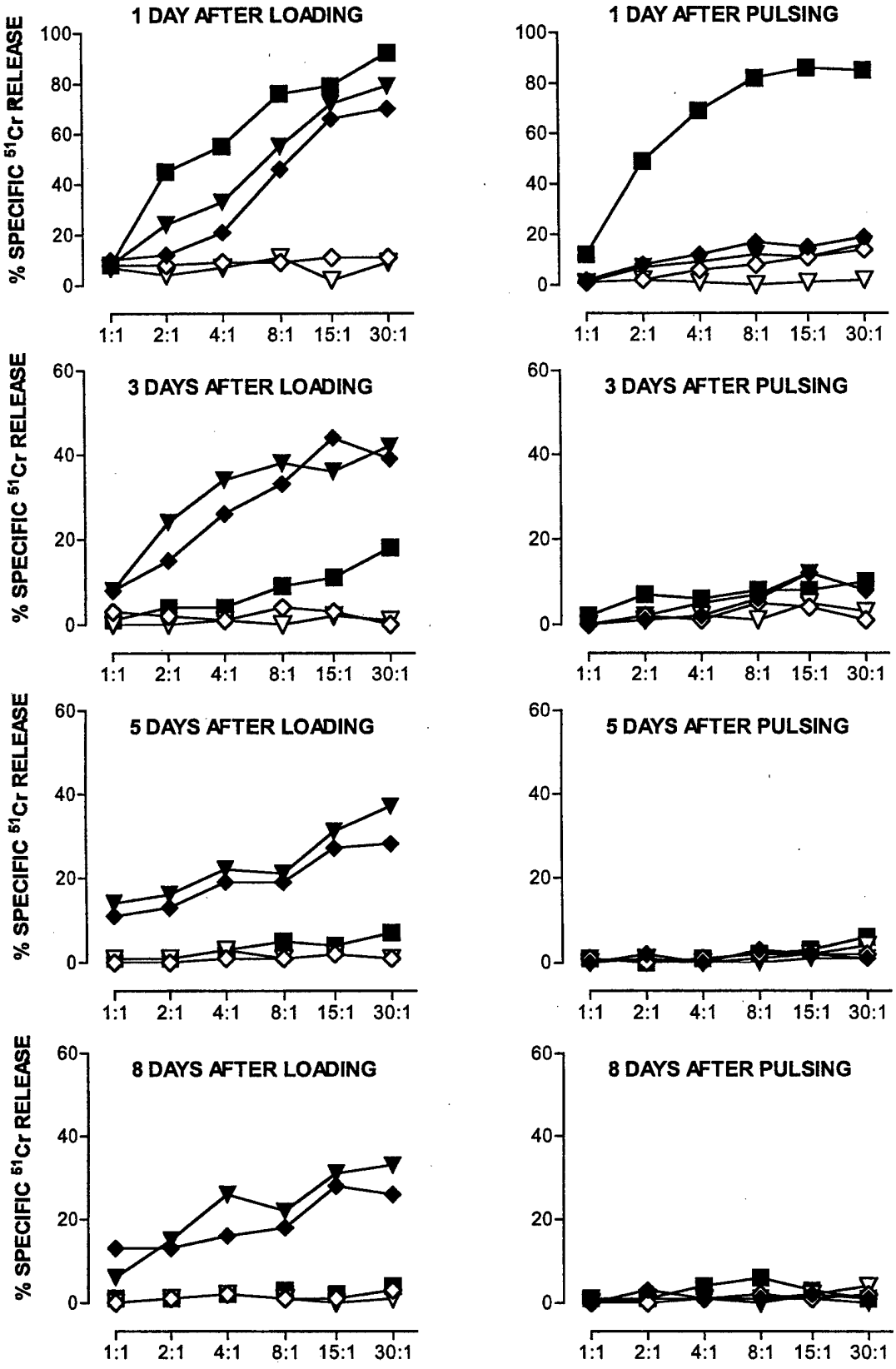
Loading/pulsing of T2 cells with peptide constructs composed of HER2/neu$_{789-797}$ incorporated into synthetic signal sequences. T2 cells were loaded (left column) or pulsed (right column) with HER$_{789-797}$-IN-AF (●), HER$_{789-797}$-IN-ES (▲), or HER$_{789-797}$ (■). At different periods after loading, T2 cells were used as targets in ^{51}Cr-release assays for the CTL.

Figure 9.



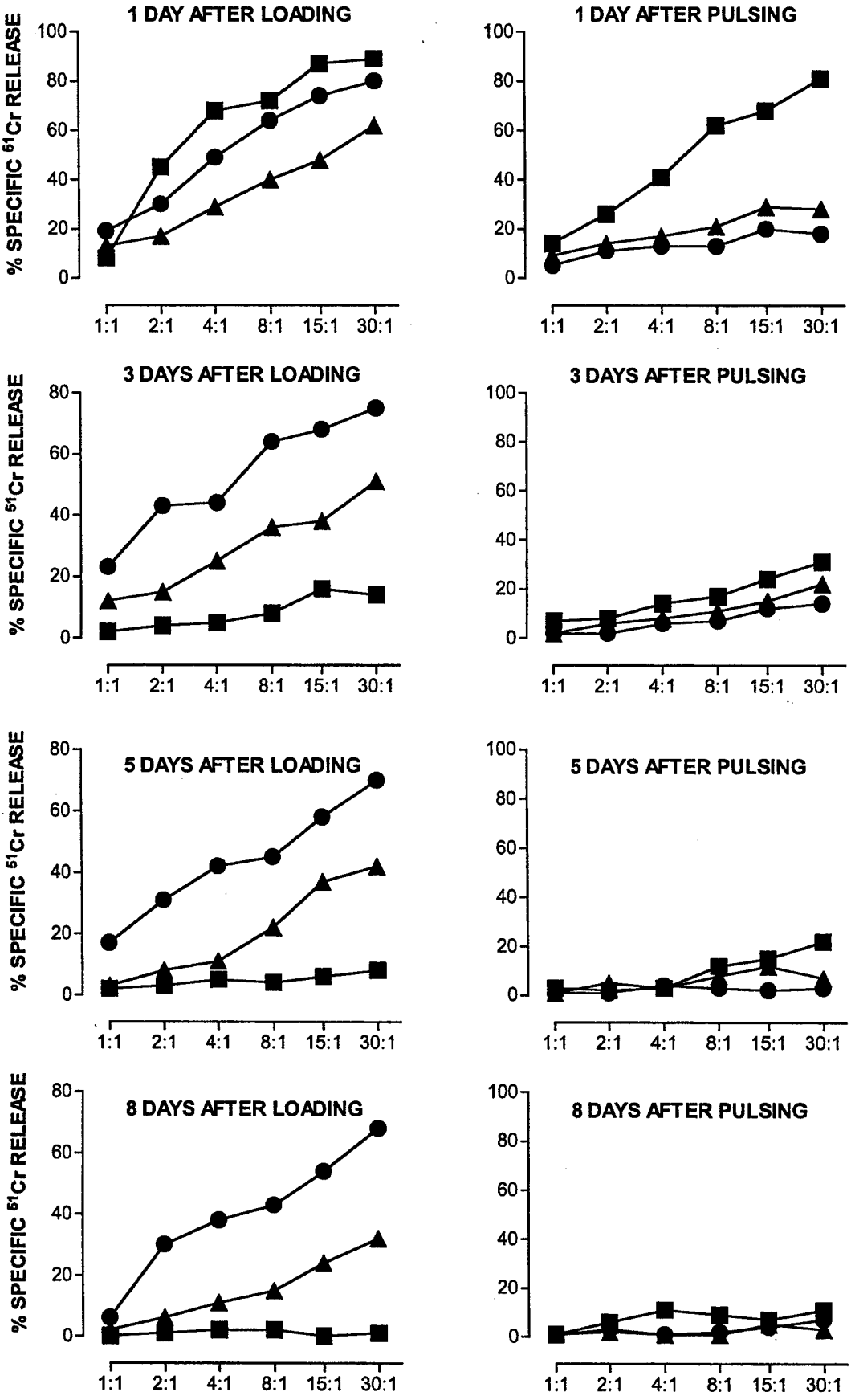
Loading/pulsing of breast cancer cells MDA-MB-231 with peptide constructs composed of synthetic signal sequences attached to amino-terminus or to carboxy-terminus of HER2/neu$_{369-377}$. MDA-MB-231 cells were loaded (left column) or pulsed (right column) with ES-HER2/neu$_{369-377}$ (◆), HER2/neu$_{369-377}$-ES (✧), IS-HER2/neu$_{369-377}$ (▼), HER2/neu$_{369-377}$-IS (∇), or HER2/neu$_{369-377}$ (■). At different periods after loading, MDA-MB-231 cells were used as targets in ^{51}Cr-release assays for the CTL.

Figure 10.



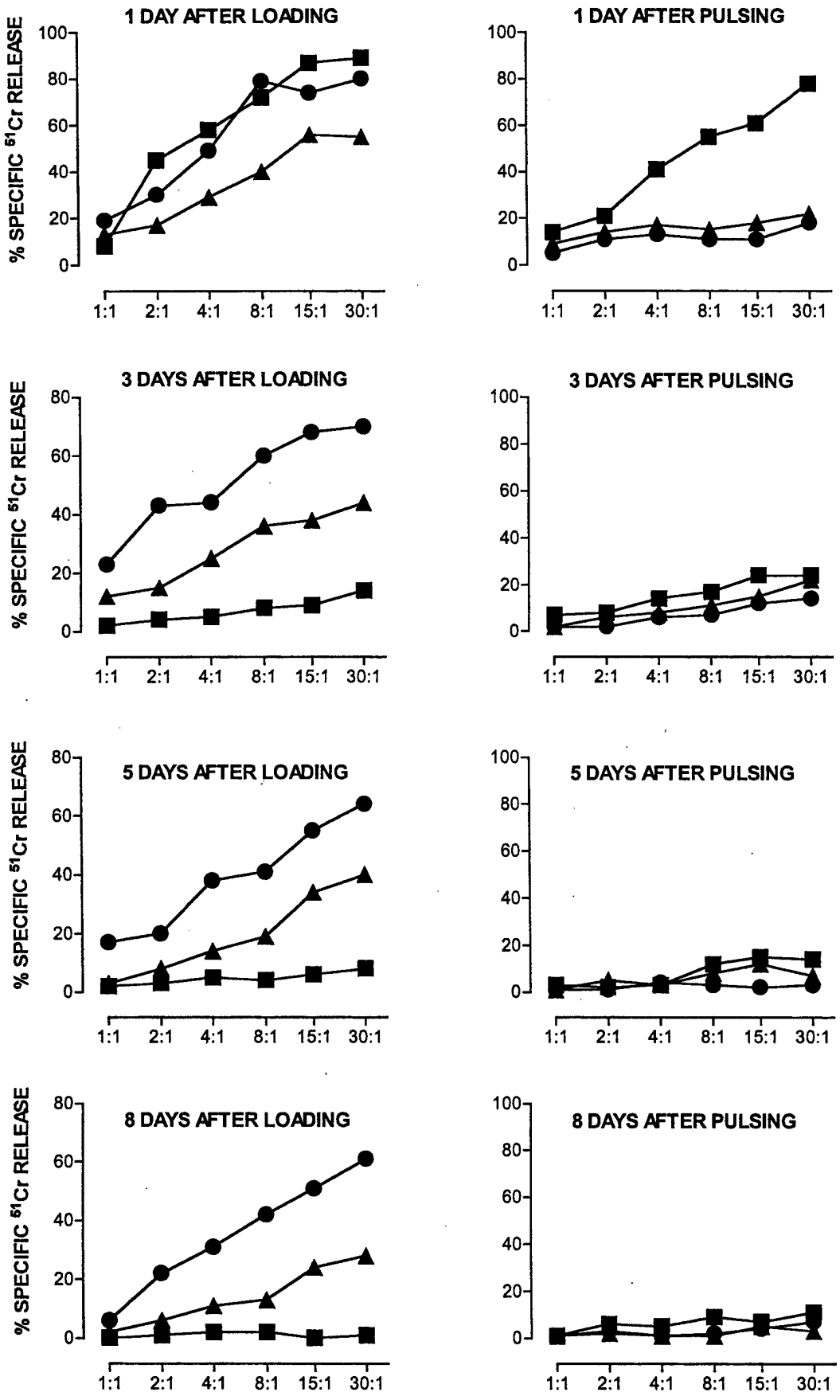
Loading/pulsing of breast cancer cells MDA-MB-231 with peptide constructs composed of synthetic signal sequences attached to amino-terminus or to carboxy-terminus of HER2/neu$_{654-662}$. MDA-MB-231 cells were loaded (left column) or pulsed (right column) with ES-HER2/neu$_{654-662}$ (◆), HER2/neu$_{654-662}$-ES (✧), IS- HER2/neu$_{654-662}$ (▼), HER2/neu$_{654-662}$-IS (▽), or HER2/neu$_{654-662}$ (■). At different periods after loading, MDA-MB-231 cells were used as targets in ^{51}Cr-release assays for the CTL.

Figure 11.



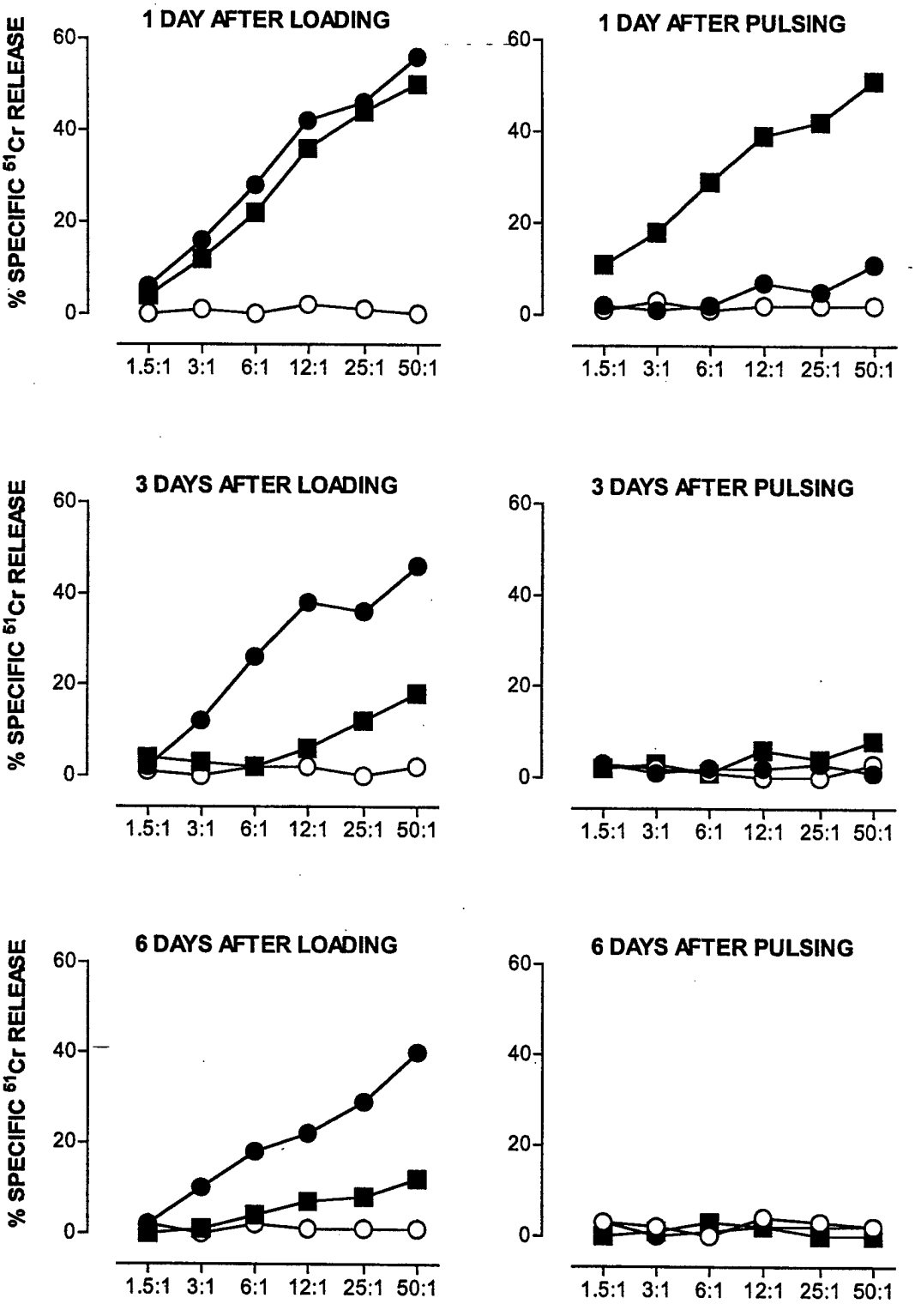
Loading/pulsing of breast cancer cells MDA-MB-231 with peptide constructs composed of HER2/neu$_{369\text{-}377}$ incorporated into synthetic signal sequences. MDA-MB-231 cells were loaded (left column) or pulsed (right column) with HER2/neu$_{369\text{-}377}$-IN-AF (●), HER2/neu$_{369\text{-}377}$-IN-ES (▲), or HER2/neu$_{369\text{-}377}$ (■). At different periods after loading, MDA-MB-231 cells were used as targets in ^{51}Cr-release assays for the CTL.

Figure 12.



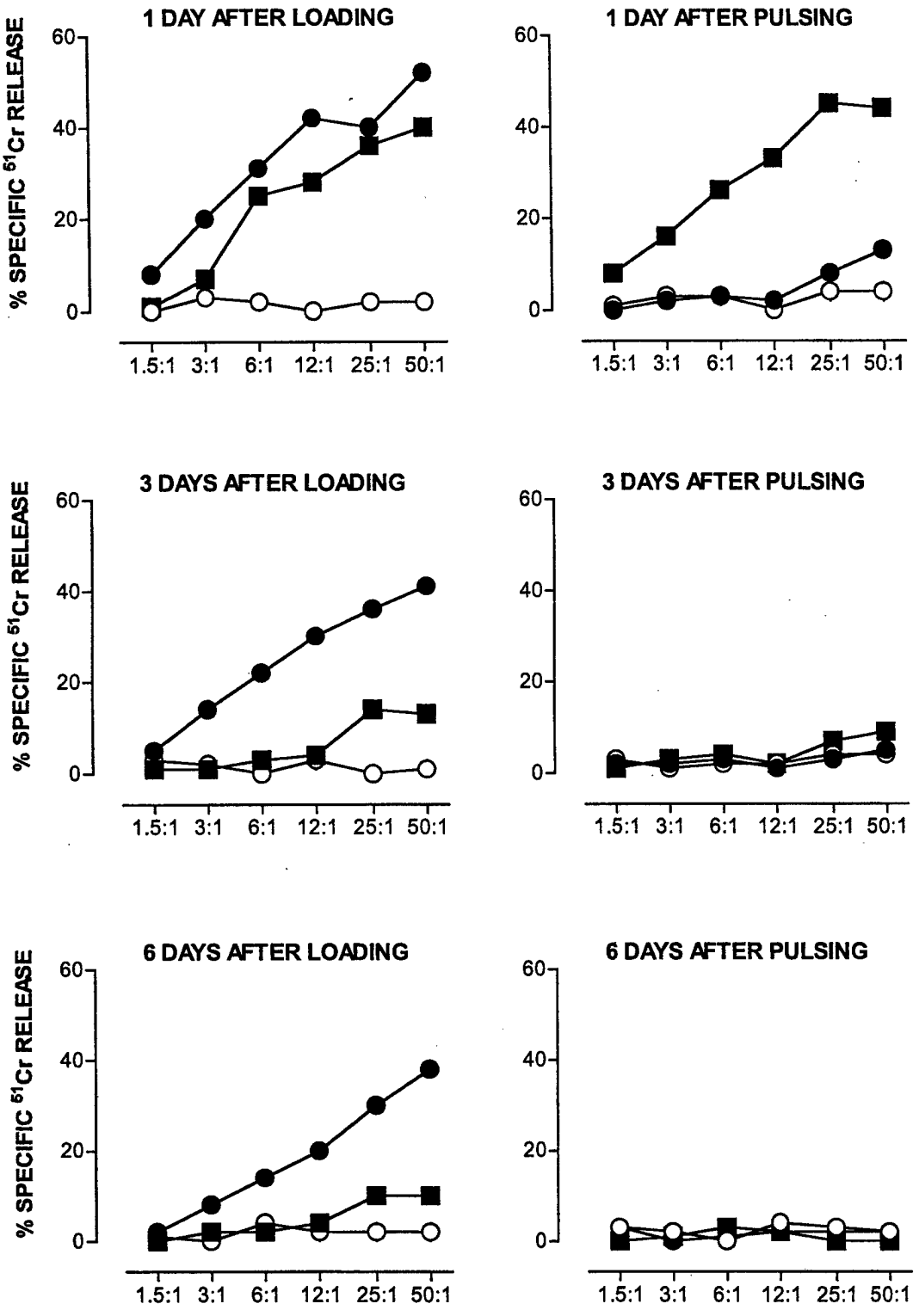
Loading/pulsing of breast cancer cells MDA-MB-231 with peptide constructs composed of HER2/neu$_{654-662}$ incorporated into synthetic signal sequences. MDA-MB-231 cells were loaded (left column) or pulsed (right column) with HER2/neu$_{654-662}$-IN-AF (●), HER2/neu$_{654-662}$-IN-ES (▲), or HER2/neu$_{654-662}$ (■). At different periods after loading, MDA-MB-231 cells were used as targets in ^{51}Cr-release assays for the CTL.

Figure 13.



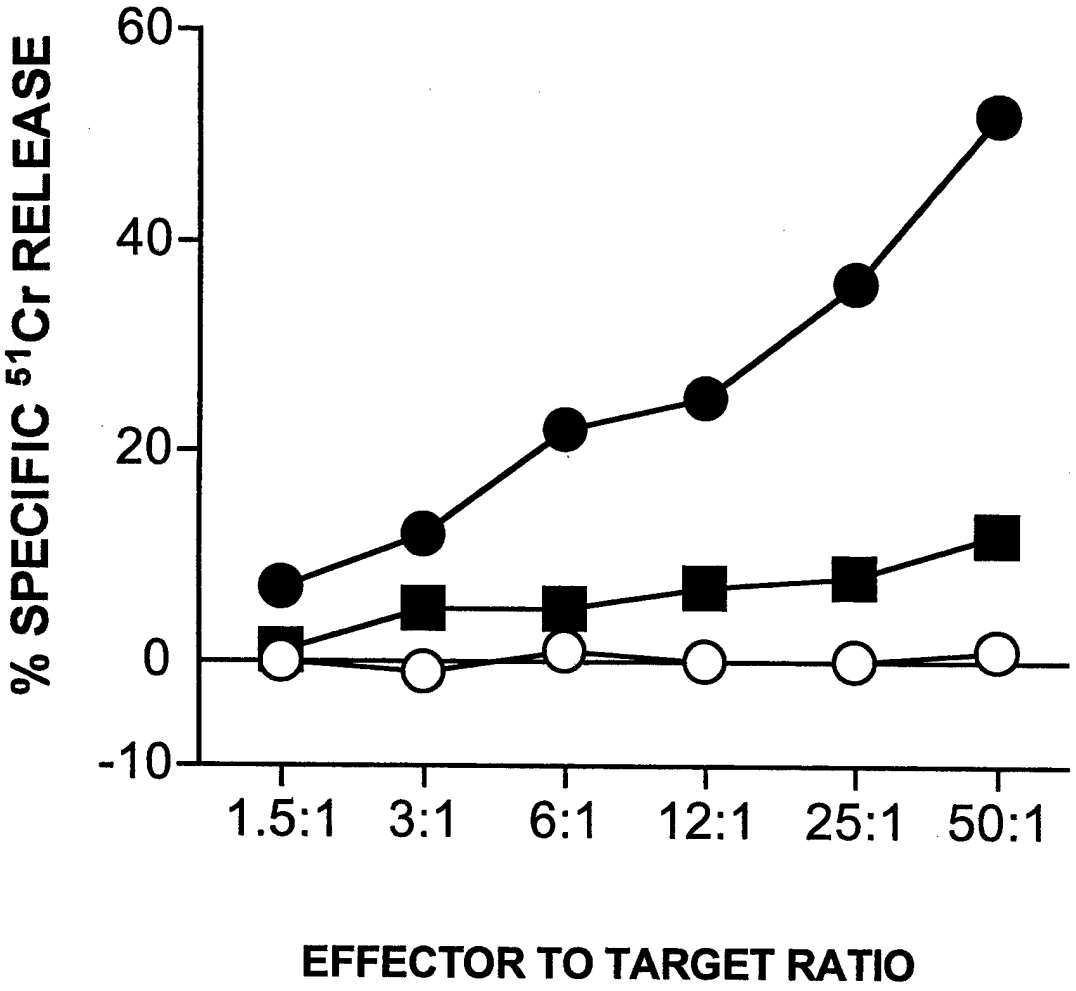
Loading of dendritic cells from healthy volunteers with peptide constructs composed of HER2/neu$_{369-377}$ incorporated into synthetic signal sequences. Dendritic cells were loaded with HER2/neu$_{369-377}$-IN-AF (●), HER2/neu$_{369-377}$ (■), or were used without loading (○). At different periods after loading dendritic cells were used as targets in ^{51}Cr-release assays for the CTL.

Figure 14.



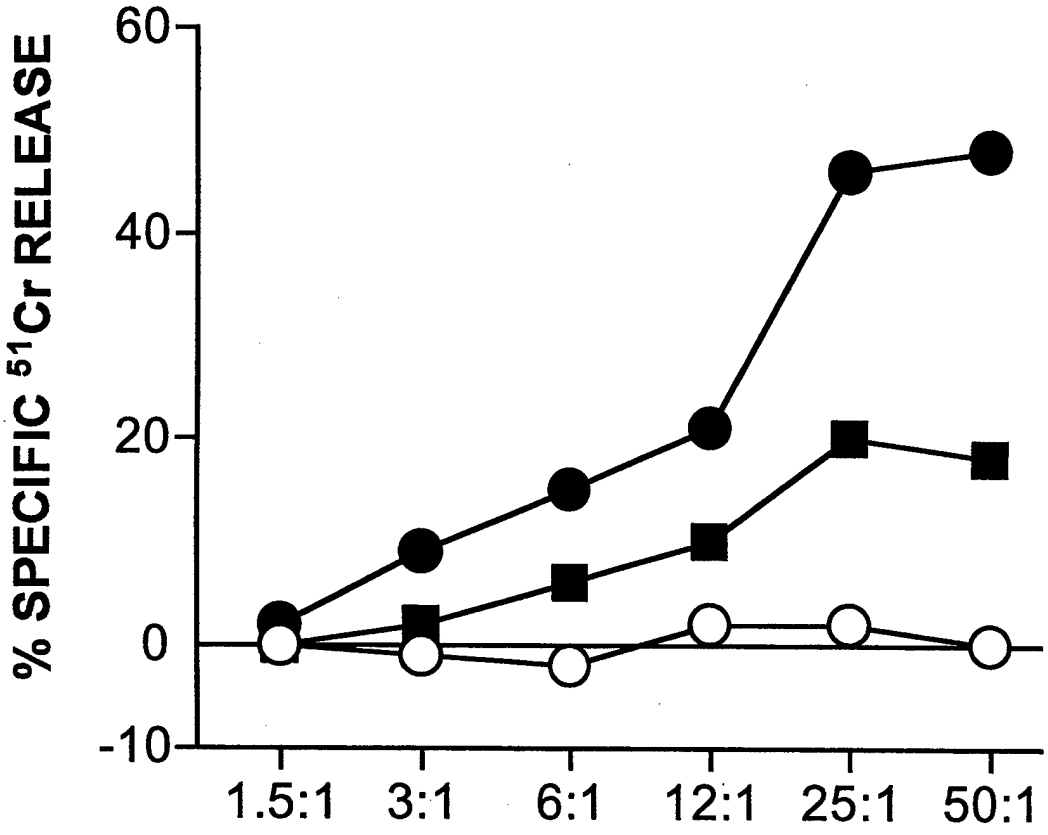
Loading of dendritic cells from healthy volunteers with peptide constructs composed of HER2/neu$_{654-662}$ incorporated into synthetic signal sequences. Dendritic cells were loaded with HER2/neu$_{654-662}$-IN-AF (●), HER2/neu$_{654-662}$ (■), or were used without loading (○). At different periods after loading dendritic cells were used as targets in ^{51}Cr-release assays for the CTL.

Figure 15.



Cytotoxic activity of CTL after two cycles of stimulation with peptide-loaded dendritic cells. Dendritic cells were loaded with HER2/neu$_{369\text{-}377}$-IN-AF (●), HER2/neu$_{369\text{-}377}$ (■), or were used without loading (○). After the second stimulation, the cytotoxic activity was measured using T2 cells pulsed with HER2/neu$_{369\text{-}377}$ as targets in ^{51}Cr-release assays.

Figure 16.



Cytotoxic activity of CTL after two cycles of stimulation with peptide-loaded dendritic cells. Dendritic cells were loaded with HER2/neu$_{654-662}$-IN-AF (●), HER2/neu$_{654-662}$ (■), or were used without loading (○). After the second stimulation, the cytotoxic activity was measured using T2 cells pulsed with HER2/neu$_{654-662}$ as targets in ^{51}Cr-release assays.

APPENDICES

INDUCTION OF CYTOTOXIC T LYMPHOCYTES FOR IMMUNOTHERAPY OF BREAST CANCER (DAMD17-00-1-0184)

1. **Manuscript:** BORIS R. MINEV, FANG GUO, IVELINA GUEORGUIEVA and HANS E. KAISER.: Vaccines for Immunotherapy of Breast Cancer and Prostate Cancer: New Developments and Comparative Aspects. *In Vivo* 15 (5), 2002 (In Press)

2. **Abstract:** BORIS R. MINEV, JASON HIPP and JENNIFER HIPP.: Development of Synthetic Vaccines for Immunotherapy of Breast Cancer. *Era of Hope Meeting,* Orlando, Florida, September 25-28, 2002

3. **United States Provisional Patent Application:** BORIS R. MINEV: Enhancing Class I Antigen Presentation With Synthetic Sequences. *UCSD Technology Transfer & IP Services,* July 11, 2003

4. **Curriculum Vitae**

in vivo *16:* 405-416 (2002)

Review

Vaccines for Immunotherapy of Breast Cancer and Prostate Cancer: New Developments and Comparative Aspects

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**International Society for the Study of Comparative Oncology, 433 South West Drive, Silver Spring, MD 20901, U.S.A.*

Abstract. *This review summarizes the most recent findings and the newest approaches in the design of vaccines for breast cancer and prostate cancer. The antigens associated with breast cancer and prostate cancer and their use in vaccine development are discussed. We consider the comparative aspects in the new vaccines and focus on their clinical potential. The future directions in designing cancer vaccines are discussed.*

Immunotherapy in human cancer began with the use of intact bacterial organisms like BCG and C Parvum and has considerably evolved over the past decade, mainly due to the discovery of tumor-associated antigens in certain tumors. The significant interest in cancer vaccines today is based on two recent advances which have allowed the design of specific vaccine approaches: improved molecular techniques for the identification of genes encoding tumor-associated antigens, and better understanding of the mechanisms involved in antigen processing, presentation, and T cell activation. T cells expressing CD4 molecules recognize peptides of 12-25 amino acids presented by MHC class II molecules (1). The cytotoxic T lymphocytes expressing CD8 molecules recognize class I restricted peptides of 8-10 residues which are the products of intracellularly processed proteins (2).

The realization that MHC class I restricted tumor antigens can act as targets for cytotoxic T lymphocytes (CTL) (3) promoted the search for tumor antigen genes (4, 5). CTL appear to be among the most direct and effective elements of the immune system that are capable of generating antitumor immune responses (6). Tumor cells expressing the appropriate tumor-associated antigens can be effectively recognized and destroyed by these immune effector cells, which may result in dramatic clinical responses (7-9). Both the adoptive transfer of tumor-reactive CTL and active immunization designed to elicit CTL responses have been reported to lead to significant therapeutic antitumor responses in patients with malignant melanoma (7-9). However, these promising approaches and their applicability to other tumor types besides melanoma are restricted because of the limited number of tumor-associated antigens or epitopes for CTL.

A variety of approaches have been used for the identification of tumor-associated antigens (TAA) recognized by CTL. Most of the melanoma antigens have been identified by screening cDNA expression libraries with CTL reactive against melanoma (6). Another approach for the identification of TAA involves testing of known proteins for recognition by CTL (10, 11). Direct isolation and sequencing of peptides eluted from the tumor cells is yet another method of identifying tumor-associated peptide antigens (12, 13). An important advantage of this technique is the direct identification of peptides naturally processed and presented on the tumor cell surface. Serological analysis of recombinant cDNA expression library of human tumors with autologous serum (SEREX) was also used to isolate human tumor antigens (14). Examples are tyrosinase, MAGE, NY-ESO-1, SSX2, SCB-1, and CT7 (6). Some of these antigens are T-cell defined antigens, which emphasizes the usefulness of SEREX analysis in identifying new tumor antigens. More recently, computer programs have been used to identify peptide sequences of known proteins based on their binding affinity for selected HLA molecules (15). We analyzed the sequence of human telomerase reverse transcriptase (hTRT) (16) for peptide sequences containing binding motifs for the HLA-A2.1 molecule by using the software of the Bioinformatics & Molecular Analysis Section (National Institutes of Health, Washington, DC, USA) (17). We demonstrated that two of the highest-ranking hTRT peptides can generate *in vitro* CTL able to recognize and kill a variety of HLA-A2+ cancer cell

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Key Words: Cancer vaccines, comparative oncology, immunotherapy, breast cancer, prostate cancer, tumor-associated antigens, antigen presentation.

lines (18). Using different computer-based algorithms, we identified six epitopes recognized by human CTL within the sequence of the new tumor-associated antigen MG50, which we described recently (19).

In this review, we will summarize the most recent advances in the development of vaccines for breast cancer and prostate cancer. Tumor-associated antigens as a basis for the development of the new vaccines will be discussed. In addition, we will discuss the comparative aspects and the future clinical applications of these vaccines.

Vaccines for Immunotherapy of Breast Cancer

Breast cancer is one of the major causes of death in women in industrialized countries. Surgery remains the mainstay of therapy for early disease. However, despite advances in chemotherapy and radiation therapy, advanced breast cancer still carries a high mortality rate. This justifies the development of vaccines for immunotherapy of breast cancer as a promising new treatment modality.

Breast cancer has been considered a nonimmunogenic tumor in contrast to melanoma and renal cell carcinoma. However, a fast growing number of reports confirm the role of the immune system in breast cancer (20, 21). Several recent studies have demonstrated the presence of breast cancer-specific cytotoxic T lymphocytes in peripheral blood from breast cancer patients (22, 23).

Early dissemination of treatment-resistant tumor cells is the major cause of metastatic recurrence in breast cancer patients (24). Micrometastatic breast cancer cells are frequently detected in the bone marrow of early-stage breast cancer patients (25), and most of these cells rest in the G0 phase of the cell cycle (26). Since chemotherapeutic agents have a reduced efficacy in non-proliferating cells (27), immunotherapy may represent a valuable treatment option because it is able to eliminate residual tumor cells independent of their proliferative state.

Tumor heterogeneity in breast cancer is another major problem in the clinical management of this disease. Breast cancers have many different populations of cancer cells expressing a variety of tumor-associated antigens (28), which may limit the efficacy of monovalent immunotherapeutic strategies directed against only one particular antigen. Thus, defining new target antigens expressed by the breast cancer cells emerges as a crucial first step in selecting appropriate therapeutic targets.

A large number of TAA have been identified in melanoma and other tumors (6, 19, 29). In contrast, very few antigens expressed in breast cancer including carcinoembryonic antigen (CEA) (30), MAGE I and MAGE 3 (31), Mucins (32), p53 (33), HER-2/neu (34), as well as several less well characterized antigens (20, 21) have been described. However, these antigens are also expressed on some normal cells, which raises concerns about the likelihood of generating clinically efficient immune responses, as well as in regard to serious autoimmune toxicity.

Ideal targets for breast cancer immunotherapy would include antigens that are: (i) highly expressed in all breast cancer cells; (ii) not expressed in normal tissues; and (iii) able to induce potent cytotoxic immune responses.

One such target might be the enzyme telomerase. Telomerase, a cellular reverse transcriptase that maintains the ends of chromosomes (telomeres), is activated in the vast majority of breast cancers (over 90% of breast carcinomas) but not in normal adjacent tissues (35, 36). Recently, we identified two HLA-A2-restricted peptides derived from human telomerase reverse transcriptase (hTRT), and induced hTRT-specific CTL *in vitro*. Importantly, we demonstrated that these CTL lysed the HLA-A2-positive breast cancer cell line MCF7, but not the HLA-A2-negative breast cancer cell line SKBR3 (18). Both cell lines were hTRT-positive as determined by the TRAPeze assay (Intergen). Further pre-clinical studies need to be performed before the initiation of clinical trials with telomerase-derived vaccines for breast cancer.

Another promising target for immunotherapy of breast cancer is the proto-oncogene HER2/neu, expressed in breast cancer and other human cancers. HER2/neu encodes a tyrosine kinase with homology to epidermal growth factor receptor (37). In breast cancer, HER2/neu overexpression is associated with aggressive disease and is an independent predictor of poor prognosis (38, 39). HER2/neu is considered a possible target for T-cell-mediated immunotherapy for several reasons: (i) the protein is large (1255 amino acids), contains epitopes appropriate for binding to most MHC molecules and thus is potentially recognizable by all individuals; (ii) HER2/neu is greatly overexpressed on malignant cells and thus T-cell therapy may be selective with minimal toxicity; (iii) the oncogenic protein is intimately associated with the malignant phenotype and with the aggressiveness of the malignancy, especially in breast and ovarian carcinomas (38-40). Indeed, many studies described existent immunity to HER2/neu in patients with breast cancer. Evaluation of large number of patients with breast cancer revealed the presence of antibodies to HER2/neu (41), helper T-cell immunity to HER2/neu (42), as well as CTL specific for HER2/neu (43). Several class I-restricted HER2/neu-derived peptides recognized by breast cancer-specific CTL have been described (43-45). We studied whether synthetic endoplasmic reticulum insertion signal sequences could improve the presentation of several class I-restricted peptides derived from HER2/neu. We utilized antigen processing-deficient T2 cells, which lack the ability to translocate peptides across the ER membrane and present them on the cell surface, and human dendritic cells. Nonamer HER-2/neu-derived peptides fused to, or included within, synthetic signal sequences were introduced into the cytosol of T2 cells and DC with a technology called "osmotic lysis of pinocytic vesicles". With standard ^{51}Cr-release assays, we tested the ability of HER-2/neu-specific CTL to recognize the peptide-loaded cells at various intervals after loading. We observed significant lysis of T2 cells and DC loaded with

peptide constructs composed of a signal sequence fused to the amino-terminus, but not the carboxy-terminus of the HER-2/neu peptides. Interestingly, T2 cells and DC loaded with the HER-2/neu peptides included within an artificial signal sequence were recognized most efficiently for at least 6 days after loading (46). This study and a growing number of other preclinical studies (45, 47, 48) suggest that HER2/neu-derived peptides could be used as specific vaccines in patients with HER2/neu-expressing tumors.

Several clinical trials evaluated different immunization strategies targeting HER2/neu. In a phase I study, patients were immunized with CD4+ T-helper peptides derived from HER2/neu. The peptides were admixed with granulocyte-macrophage colony-stimulating factor as adjuvant and administered intradermally once monthly for six months. The toxicity of the vaccine was minimal and the majority of patients developed peptide-specific and protein-specific immunity (49). Interestingly, immunity to epitopes not included in the immunizing mix was induced. This finding suggests that the immune repertoire to HER2/neu develops during the course of vaccination. Another clinical trial was aimed at inducing CTL against HER2/neu using a single HLA-A2-restricted epitope (50). In this trial, immunization resulted in the generation of HER2/neu peptides-pecific interferon γ-producing CTL. These CTL, however, were not able to lyse or produce cytokines when exposed to HER2/neu-expressing tumors. In a more recent trial, patients were immunized with HER2/neu-derived helper peptides that encompassed CTL epitopes within the natural protein sequence (51). An increase of the HER2/neu peptide- specific T-cell precursor frequency was observed in the vaccinated patients. These promising clinical studies show that patients with HER2/neu-expressing tumors can be immunized with HER2/neu-derived peptides and are able to develop measurable immunity against this protein. Another strategy for immunization was attempted recently in breast cancer patients. The patients were immunized with modified allogeneic tumor cells, which are HLA-A2 restricted, express HER2/neu, and were transfected to express the costimulatory molecule CD80 (52). Another recent study was designed to assess the safety and the efficacy of autologous dendritic cells loaded with HER2/neu peptide (53). These important studies, when carefully analyzed, will help in deriving effective vaccination strategies targeting HER2/neu.

The tumor-associated antigen MUC-1 is abundantly over-expressed on the cell surface of many human adeno-carcinomas, such as breast, ovarian, prostate and colon cancers, and hematological malignancies. It is therefore a suitable candidate for broadly applicable vaccine therapies (54, 55). MUC-1 is a highly glycosylated type I transmembrane glycoprotein with a unique extracellular domain consisting of a variable number of tandem repeats (VNTR) of 20 amino acids (PDTRPAPGSTAPPAHGVTSA) (56). It was demonstrated that the MUC-1 protein expression and secretion in cancer patients is associated with high metastatic potential and poor prognosis (57-59). Several *in vitro* studies have shown that the MUC-1 protein can induce apoptosis or inhibit T-cell proliferation (60, 61). These effects were mediated by the whole MUC-1 protein or large synthetic tandem repeats of the MUC-1 core peptide and were reversible by addition of IL-2, anti-CD28 monoclonal antibody, or short 16-amino acid MUC-1 peptide (61). This indicates that active specific immunotherapy using short synthetic peptides in combination with professional APC and/or IL-2 may overcome the observed immunosuppression in cancer patients. It was demonstrated that MUC-1 reactive T cells found in patients with breast cancer, pancreatic cancer, and multiple myeloma recognize the MUC-1 molecule in an MHC-unrestricted manner (62, 63). However, there is increasing evidence that MHC-restricted T cells can also be induced in mice and humans after immunization with the MUC-1 protein or MUC-1 antigenic epitopes (64-68). Several MHC class I-restricted MUC-1 -derived peptides recognized by CTL have been identified. Two novel HLA-A2-restricted peptides, M1. 1 and M1.2, were recently identified (69). The M1.1. 1 peptide (STAPPVHNV) was derived from the VNTR domain of the MUC-1 protein, whereas the M1.2 peptide (LLLLTVLTV) was located in the leader sequence. CTL induced with these peptides efficiently lysed target cells pulsed with the corresponding peptide, or tumor cells naturally expressing MUC-1 in an MHC-restricted and antigen-specific manner (69). More recently, the authors demonstrated that patients with advanced breast and ovarian cancer can be efficiently vaccinated with autologous dendritic cells pulsed with these MUC-1 -derived peptides. A significant CTL response in the cancer patients was induced with the peptide M1.2, which lasted for more than 6 months (53). A promising vaccine for breast cancer and other human cancers called Theratope (®) has been designed by Biomira, Inc. Theratope (®) consists of a synthetic mimic of the mucin-associated glycan epitope STn conjugated to the carrier molecule KILH (keyhole limpet hemocyanin) (70). The vaccine has been shown to stimulate anti-STn antibodies and mucin-specific T-cell responses, which were augmented by pretreatment with cyclophosphamide used to inhibit suppressor T cells (71-73). Several clinical studies suggested that Theratope (®) is well-tolerated in breast and ovarian cancer patients, and the trends in data suggest that Theratope (®) may decrease the risk for relapse and death (74, 75). Other trials showed that MUC-1 -derived peptide vaccines could induce specific CTL responses (66), and antibody responses (76) against MUC-1 in breast cancer patients. These important clinical studies suggest that MUC-1 could be a suitable target for immunotherapy of breast cancer.

Vaccines for Immunotherapy of Prostate Cancer

Prostate cancer is the most frequently diagnosed cancer and the second leading cause of cancer death in men in North America (77). Although locally confined disease is treatable,

recurrent and metastasized prostate cancer is essentially incurable. Androgen ablation therapy may palliate advanced disease, as some prostate cancer cells are androgen-responsive (78, 79). However, the majority of patients inevitably progress to incurable, androgen-independent disease (80, 81). Tumor heterogeneity in prostate cancer is a major problem in the clinical management of this disease. Prostate tumors have many different populations of cancer cells expressing a variety of tumor-associated antigens (TAA). In addition, the progression of prostate cancer from the hormone-naive primary to increasingly androgen-independent metastatic lesions is associated with a number of molecular and genetic changes, which affect the expression of specific TAA on the cell surface.

Several antigens expressed in prostate cancer including prostate-specific antigen (PSA) (82), prostate-specific membrane antigen (PSMA) (83, 84), prostate carcinoma tumor antigen (PCTA-1) (85), prostatic acid phosphatase (PAP) (86), the gene product of the metastasis-suppressor gene on human chromosome 11p 11.2 (KAI 1) (87), and prostate stem cell antigen (PSCA) (88), have been described. Telomerase activity has been also associated with prostate cancer but not with normal prostatic tissue. Telomere lengths were also found to be consistently shorter in cancer tissue (89). These results suggest that, as in other cancers, telomerase activity may be a marker for prostate cancer. We demonstrated recently that the majority of normal individuals and patients with prostate cancer immunized *in vitro* against two telomerase-derived peptides could elicit tumor-specific CTL. Most importantly, the CTL of prostate cancer patients specifically lysed a variety of cancer cell lines, demonstrating immunological recognition of endogenously processed telomerase peptides (18). In order to effectively treat the very heterogeneic prostate cancers, it is critical to identify novel markers and therapeutic targets in advanced prostate cancer and androgen-independent disease. Ideal targets for prostate cancer immunotherapy would include proteins that are: (i) highly expressed in metastatic prostate cancer cells; (ii) not expressed, or expressed at very low level in normal tissues; (iii) accessible to therapeutic modalities at the cancer cell surface; (iv) not modulated by androgen.

The identification of tumor antigens and proteins involved in prostate cancer progression, and the promising vaccine studies in animal models support the design and clinical testing of new prostate cancer vaccines.

In a recent clinical study sixty patients with hormone-refractory prostate cancer were treated with a combination of irradiated allogeneic prostate cancer cells and the adjuvant SRL 172 (90). Although there was no significant decline in PSA from the entry level, several patients had an increase in cytokine production, increases in specific antibodies and evidence of T-cell proliferation in response to the vaccinations. Importantly, the vaccine was safe and well-tolerated suggesting that further exploration of the use of whole-cell allogeneic vaccines for immunotherapy of less advanced prostate cancer may yield better clinical responses.

Another approach to tumor vaccination involves irradiated tumor cells modified with cytokine genes to secrete these cytokines. Twenty-one patients with recurring micrometastatic prostate cancer were vaccinated with irradiated allogeneic cancer cells secreting granulocyte-macrophage colony-stimulating factor (GM-CSF) (91). One of the patients had a partial response lasting more than seven months, 14 out of 21 patients had stable disease and 6 patients progressed. Importantly, 71% of the patients had a decreased PSA level compared with the one before vaccination. This study confirmed that GM-CSF transduced allogeneic vaccines can break the immune tolerance of prostate cancer and induce tumor-specific immunity.

In another clinical trial, patients with prostate cancer were vaccinated with the hexasaccharide globo H conjugated to KLH (92). Although clinical response was not observed, this vaccine was able to induce a specific high titer of IgG and IgM antibodies against globo H. This vaccine has a potential for treatment of prostate cancer because it can enhance the B-cell immunity.

A phase I trial exploring the immunogenicity of the ganglioside GM2 conjugated to KLH in patients with relapsed prostate cancer has been performed (93). Patients received the vaccine at weeks 1,2,3,7 and 19. This vaccine was found to be safe and all patients generated high-titer IgM and IgG antibodies with specificity for GM2. All patients in the study with metastatic disease except for the 1 patient with a single involved lymph node showed radiographic and biochemical progression of disease. The patient with the single lymph node involvement had radiological evidence of decrease in lymph node size by 50% as well as stabilization of PSA levels.

As we discussed in the breast cancer section of this review, the tumor-associated antigen MUC-1 is abundantly over-expressed on the cell surface of many human adeno-carcinomas, including prostate cancer, and is therefore a suitable candidate for broadly applicable vaccine therapies (54, 55). A MUC-1 -derived vaccine composed of a peptide conjugated with KLH, was injected with the immune adjuvant QS21 in patients with prostate cancer (94). All patients generated IgM and IgG response after three immunizations, and the rate of PSA increase diminished in some patients (95).

Prostate cancer recurrence, evidenced by rising PSA levels after radical prostatectomy, is an increasingly prevalent clinical problem. Prostate-specific antigen (PSA)-based vaccine was able to induce PSA-reactive effector cells after vaccination of patients with prostate cancer (96). The vaccine consisted of recombinant PSA with lipid A formulated in liposomes. This vaccination induced T-cell responses in 8 of 10 patients with prostate cancer. The observed cellular responses were predominantly mediated by CD4+ T lymphocytes. Another clinical study was undertaken to evaluate the safety and biological effects of vaccinia -PSA (PROSTVAC) administered

to 6 patients with post-prostatectomy recurrence of prostate cancer (97). Toxicity was minimal, and primary anti-PSA IgG antibody activity was induced after vaccinia-PSA immunization in one subject, although such antibodies were detectable in several subjects at baseline. Eder *et al.* recently performed a phase I trial of a recombinant vaccinia virus expressing PSA in patients with advanced prostate cancer (98). Thirty-three patients received the vaccine at 4-week intervals for a total of three doses. Stable disease was reported in 14 out of 33 patients for at least 6 months. In five of the seven HLA-A2-positive patients the authors observed an increase of PSA peptide-specific T-cell frequencies. Interestingly, the T-cell increases were only observed after the first vaccination, and T cells did not further increase with subsequent vaccinations. This study demonstrated the safety and feasibility of the recombinant vaccine approach.

These early clinical studies with synthetic and recombinant vaccines for prostate cancer are very encouraging. In contrast to other vaccine vectors, viruses elicit strong and long-lasting immune response, and are able to infect nearly all host cells, as well as to ensure intracellular translation, degradation and efficient trafficking of peptide antigens to the cell surface. The potential drawbacks of the viral vectors are related to their safety and pre-existing immunity, particularly to vaccinia virus and adenoviruses. However, the safety of the viral vaccines can be ensured by using non-replicating, highly attenuated or genetically modified viruses, while the problem of pre-existing immunity may be circumvented by the use of non-mammalian viruses, such as the avian poxviruses. Therefore, the use of recombinant viruses as cancer vaccines is very promising.

A growing number of studies report the successful use of dendritic cells (DC) for inducing anti-tumor immune responses in both animals and patients. DC are the most potent antigen-presenting cells for the initiation of antigen-specific immune responses (99). In addition to their ability to efficiently acquire and process antigens (100), DC express high levels of MHC class I and class II molecules as well as costimulatory molecules (101), essential in antigen presentation. Therefore, many investigators attempted to immunize with peptide-pulsed DC. It was found that immunization with peptide-pulsed DC is superior to injection of peptide in adjuvant in inducing potent cytotoxic T-cell responses (102).

The encouraging results in experiments in mice (103), as well as improved techniques for isolation and purification of DC (104), support the initial attempts to immunize patients with DC-expressing tumor antigens. Valone *et al.* (105) have carried out a trial of partially purified peripheral blood DC pulsed with recombinant PAP protein in 12 patients with advanced prostate cancer. Intravenous administration of peptide-pulsed DC monthly for 3 months resulted in T-cell proliferative responses to PAP in all patients. Murphy *et al.* assessed, in a phase I clinical trial, the safety of administering autologous DC pulsed with HLA-A2-restricted PSMA peptides in 51 patients with advanced androgen-independent prostate cancer (84). Toxicity of the treatment was not observed, except for mild hypotension during the time of infusion. Patient clinical response was analyzed based on National Prostate Cancer Patient (NPCP) criteria and a minimum of 50% reduction of serum PSA levels. Seven partial responders were observed. Average PSA levels showed a significant increase in the non-responder group, while a decrease was observed in the seven partial responders (106). The same group presented very promising results in a phase II trial with prostate cancer patients. All study participants received six infusions of autologous DC pulsed with the PSMA peptides at 6-week intervals. With each infusion, half of the study subjects received a 7-day course of subcutaneous injection of GM-CSF as systemic adjuvant. Based on the NPCP criteria and 50% reduction in PSA, 27% of the patients were identified as partial responders and 33% exhibited no significant change during the phase II trial (107). Twelve out of 19 subjects (63%) with stage D_2 hormone-refractory metastatic prostate cancer survived for more than 600 days (median survival, 608 days) (106). No significant difference in clinical response was observed in patients who received subcutaneous GM-CSF injection with their DC/peptide infusion as compared to those who only received DC/peptide infusions (108). To evaluate whether the responses were durable or not, study participants were afforded periodic follow-up evaluations after the conclusion of the study. A majority of the responders (11 out of 19) (58%) were still responsive at the end of the current follow-up (109). This study suggests the majority of the responses identified in the various groups appear to be durable. The same researchers recently reported the immune monitoring of a phase II clinical trial in prostate cancer patients before and after immunotherapy with DC exogenously pulsed with two PSMA-derived peptides (110). Clinical responses were strongly associated with 2 indicators of immunocompetence: skin test responses to recall antigens and cytokine secretion by T cells after non-specific stimulation. These authors also reported that infusions with PSMA-pulsed DC can be given with greater numbers of DC and a lesser number of infusions, with no loss of response rates (111).

To explore the potential role of xenoantigen immunization in prostate cancer patients Fong *et al.* recently performed a phase I trial using DC pulsed with recombinant mouse PAP (mPAP) as a vaccine (112). Twenty-one prostate cancer patients were immunized twice with mPAP-loaded DC four weeks apart. All patients, developed T cell immunity to mouse PAP, and 11 of the 21 patients also developed Th1 responses to the homologous self-antigen. Importantly, 6 of the 21 patients experienced stabilization of disease as assessed by serum PSA and confirmed with radiographic imaging. This study represents the first demonstration that xenoantigen immunization can generate immune response against self-antigens in humans, resulting in a clinically significant antitumor effect.

The same antigen, PAP, was used in two sequential phase I

and phase II clinical trials in patients with hormone-refractory prostate cancer (113). The vaccine called Provenge (Dendreon Corp., Seattle, WA, USA) consists of autologous DC loaded *ex vivo* with a recombinant fusion protein consisting of PAP linked to GM-CSF. Twelve patients were treated in the phase I trial, and 19 patients were enrolled in the phase II trial. All patients tolerated the treatment well, with fever as the most common adverse effect occurring in 14% of the infusions. All patients developed T-cell proliferation responses, and 38% of patients developed immune responses to PAP. The specificity of this therapy was demonstrated by the fact that treatment with Provenge did not increase the patients' response to the recall antigen influenza. Importantly, six patients had a decline in the PSA levels. There was a correlation between the deve-lopment of an immune response to PAP and the time to disease progression.

Heiser *et al.* have shown recently that monocyte-derived DC from prostate cancer patients transfected with PSA mRNA are capable of stimulating antigen-specific CTL responses *in vitro* (114). The same group performed subsequently a phase I trial to evaluate the safety and efficacy of this approach in patients with metastatic prostate cancer (115). This trial demonstrated that the administration of PSA RNA-transfected DC stimulated the induction of PSA-specific T-cell responses without toxicity in all study patients. In addition, vaccination was associated with a decrease in the log serum PSA slope in six out of seven patients. A transient clearance of circulating tumor cells was also observed, suggesting some impact on tumor progression. This study provides evidence on the safety and *in vivo* bioactivity of RNA-transfected DC in patients with metastatic prostate cancer. Therefore, the use of DC transfected with RNA-encoded antigens may allow stimulation of specific CTL from PBMC of virtually all cancer patients independent of their MHC type, in contrast to the peptide-based vaccines, which are limited to certain patient subsets.

Mincheff *et al.* recently developed a protocol for *in vivo* transfection of DC by naked DNA and adenoviral immunization (116). This group has completed phase I and phase II clinical trials using this approach for immunotherapy of prostate cancer. Prostate cancer patients were immunized with the plasmid PSMA/CD86 encoding the extracellular portion of the PSMA and the co-stimulatory molecule CD86. Three months later, the patients were immunized with the recombinant adenovirus Ad5PSMA, encoding PSMA. All immunizations were well-tolerated with no abnormal vital signs or laboratory findings. All patients showed a positive DTH reaction at the site of the plasmid application, and none of the patients developed antibodies to PSMA. Nine of the 12 patients with advanced local prostate cancer responded to combined hormone and immune treatment, while 6 of the 18 patients with distant bone metastases responded with reduction of bone pain and at least a 50% fall in PSA (117). Although these trials showed promising results, future studies are needed to evaluate the benefits of this approach.

These clinical trials suggest that prostate cancer may be responsive to DC-based immunotherapy. However, the source of the DC for vaccination and the frequency of the CTL precursors in cancer patients should be carefully evaluated. In patients with a low frequency of peptide-specific precursors, the efficient activation of antigen- specific CTL required the use of peptide-loaded CD34+-derived, but not monocyte-derived, DC (118). This suggested that DC derived from CD34+ cells and monocytes were not functionally equivalent for the activation of CTL in patients with a low CTL precursor frequency. Another important aspect is the establishment of the optimal route of DC administration. In a recent study, three patient cohorts were immunized with antigen-pulsed DC by *i.v.*, intradermal (*i.d.*), or intralymphatic (*i.l.*) injection (119). It was determined that all patients developed antigen-specific T cell responses regardless of route, while an induction of IFN-γ production was seen only with *i.d.* and *i.l.* routes of administration. *I.v.* administration, however, was associated with a significantly higher frequency and titer of antigen-specific antibodies. This study suggests that the route of administration may affect the quality of the immune response.

Comparative Aspects

A brief review of some comparative factors of the neoplastic development and the evolution of the immune system is provided as a background to the discussed approaches to vaccination.

The class of mammals to which man belongs is biologically characterized by the mammae (breast glands). Mammals are composed of 28 orders, 146 families, 1192 genera and 4809 species (120). The comparative method showed us a remarkable diversity of the mammary gland dealing with the presence of variability of species, the morphology, physiology, immunology, connection with concomitant diseases, *etc.* It must be emphasized that the mammary glands are always present. This is in contrast to the distribution of the prostate gland in male mammals. Sea cows (*Sirenia*) do not possess prostate gland. In carnivores the prostate gland has an especially well-developed structure and is separated in two bilateral lobes. In the cat these lobes are located in the lateral and dorsal aspect of the urethra. In the stallion, only an external portion is present. The well-developed internal portion of the prostate surrounds the urethra in the bull. The prostate is u-shaped in the ram and plate-like in the boar. These few data indicate the comparative variability of this organ (121).

Non-mammalian species are used more often in comparative studies for economical reasons (122) (123). These studies open new avenues of otherwise impossible insights for research. Examples are research on the distribution of the lymphatic structures (124), bioassays in carcinogenesis (125), and studies on preneoplasia (126).

All invertebrates and vertebrates protect themselves from

invasion of microorganisms, parasites, viruses and even cells from individuals of the same species. Before any immune response can be elicited, recognition of an external signal must be achieved. The precise mechanisms of recognition allow for discrimination between self and non-self. The science of immunity is a widely integrated field. Some mechanisms of resistance comparable to the immunity in animals and man can be observed even in vascular plants. The taxonomically lowest invertebrate species - the sponges - also show clearly an antagonism toward foreign compounds (127). The immune responses in invertebrates do not involve clonal amplification of the cells producing effector molecules, there are no specific memory responses, and no somatic generation of a repertoire of receptors (128) (129).

Knowledge of the conserved and the divergent aspects of the immune systems throughout evolution is very important in understanding the unique characteristics of the immune responses in vertebrates. In contrast to invertebrates, clonal amplification of cells carrying a unique receptor generated somatically is at the core of the vertebrate immune system (130) (131). In higher vertebrates, such as birds and mammals, there are two receptor systems: B cells, which utilize immunoglobulins to bind defined antigens, and T cells, which utilize T-cell receptors (TCR) to respond to antigens in the form of processed peptides bound to surface MHC molecules. It is likely that TCR and MHC molecules evolved together early in the vertebrate lineage. Genes that encode immunoglobulins were isolated from the horned shark in the 1980s (132). Interestingly, all three types of MHC loci found in higher vertebrates (class I, class IIA and class IIB) have been found in the shark (133). These findings suggest the existence of T cells with functional TCRs in the shark. It is likely that TCRs at some point in evolution were able to recognize antigens in the absence of MHC molecules. As a result of the evolution of the immune system, only mammals are able to mount the highest quality immune responses, characterized by an increase in antibody affinity towards the antigens during the progression of the response.

Future Directions

Prostate cancer and breast cancer remain leading causes of death for men and women despite the fact that systemic chemotherapy and other treatment modalities may induce clinical responses initially. Therefore, development of new treatment approaches, such as immunotherapy, is essential. The growing number of TAA identified in breast cancer and prostate cancer becomes a solid basis for vaccine development. However, the antigenic profile of these tumors is very complex, and consists of many peptides originating from various classes of protein. This fact should be considered carefully in designing cancer vaccines. Most recent vaccine studies have focused on class I-restricted antigens as targets for cancer-specific CTL. The characterization of class II-restricted antigens as targets for CD4+ T cell responses will allow concurrent immunization with class I and class II epitopes in order to generate more potent immune responses. In any case, the ideal vaccine most likely will consist of a cocktail of tumor antigens or proteins. Very important also is the dose of antigen and the speed of antigen release in the vaccine formulations. High doses of antigen released faster may induce T-cell tolerance (134). Immune tolerance may be due to fast expansion and subsequent elimination of specific T-cell clones, or to apoptosis induced by repeated stimulation of already stimulated T-cells in the cell cycle (135). Therefore, it is essential to select as immunogens those epitopes against which tolerance has not been induced (136).

Future cancer vaccine strategies will most likely focus on more potent approaches for immunization. The use of the entire antigenic proteins might well be superior to peptide vaccines. A whole protein may provide several T-cell epitopes presented by different MHC molecules. An additional advantage of the whole protein vaccines may be the induction of humoral immune responses (137). Alone, or in conjunction with surgery, radiotherapy and/or chemotherapy, immunotherapy of prostate cancer and breast cancer can be effective in eliminating micro-metastases, in decreasing the immunosuppressive effects of the chemotherapy or radiotherapy, and in increasing the resistance to viral or bacterial infections frequently occurring in cancer patients. Many challenges exist in the development of safe and effective vaccines for prostate cancer and breast cancer. Cancer cells can undergo genetic alterations that result in loss of antigen expression or loss of the ability to present the tumor antigens. Recent advances in the design of polyvalent vaccines targeting several antigens may solve this problem. In addition, the possibility to treat patients with vaccines earlier in the course of the disease and to combine vaccines with other treatment modalities may also improve the vaccine efficacy. Further understanding of the mechanisms of the antitumor immune responses will provide a basis for improvement of cancer vaccine approaches in the future. As a result, immunotherapy may become a major treatment modality of breast cancer and prostate cancer.

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DEVELOPMENT OF SYNTHETIC VACCINES FOR IMMUNOTHERAPY OF BREAST CANCER

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Cytotoxic T lymphocytes (CTL) play an important role in eradicating tumor cells. CTL recognize minimal peptides of 8-10 residues, presented at the cell surface by MHC class I molecules. Cytosolic peptides are transported across the endoplasmic reticulum (ER) membrane with the help of the ATP-dependent transporters associated with antigen processing (TAP). Peptides complexed with class I molecules in the ER are then transported to the cell surface for recognition by CTL. The transport into the ER can also be accomplished via insertion signal sequences located at the NH2-terminus of the precursor molecules.

We studied whether synthetic endoplasmic reticulum insertion signal sequences could improve the presentation of class I-restricted peptides derived from the shared tumor antigen HER2/neu. We utilized antigen processing-deficient T2 cells, which lack the ability to translocate peptides across the ER membrane and present them on the cell surface, and human dendritic cells (DC). Nonamer HER2/neu-derived peptides fused to, or included within, synthetic signal sequences were introduced into the cytosol of T2 cells and DC with a technology called "osmotic lysis of pinocytic vesicles". With standard 51Cr-release assays and interferon gamma ELISA assays, we tested the ability of HER2/neu-specific CTL to recognize the peptide-loaded cells at various intervals after loading.

We observed significant lysis of T2 cells and DC loaded with peptide constructs composed of a signal sequence fused to the amino-terminus, but not the carboxy-terminus of the HER2/neu peptides. Interestingly, T2 cells and DC loaded with the HER2/neu peptides included within an artificial signal sequence were recognized most efficiently for at least 6 days after loading. T2 cells and DC loaded with the minimal peptides were only marginally recognized. The efficiency of the signal sequences in facilitating the HER2/neu peptide presentation was confirmed also by using interferon gamma ELISA assays.

Signal sequences combined with minimal antigenic peptides can improve antigen-presentation of tumor antigens and stimulate CTL more efficiently. This novel approach may be of practical significance for the development of synthetic vaccines for immunotherapy of breast cancer.

The U.S. Army Medical Research and Materiel Command under DAMD17-00-1-0184 supported this work.

The Department of Defense Breast Cancer Research Program Meeting

Era of Hope

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Docket Number **UCSD1640**

PROVISIONAL APPLICATION FOR PATENT COVER SHEET

This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 CFR 1.53 (c).

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TITLE OF INVENTION (280 characters max)

ENHANCING CLASS I ANTIGEN PRESENTATION WITH SYNTHETIC SEQUENCES

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ENCLOSED APPLICATION PARTS (*check all that apply*)

X	Specification	*Number of Pages*	24		X	Entitled to Small Entity Status	Yes
X	Drawing(s)	*Number of Sheets*	15		X	Other (*specify*)	Tables (8 pages)

METHOD OF PAYMENT OF FILING FEES FOR THIS PROVISIONAL APPLICATION FOR PATENT (*check one*)

X	A check or money order is enclosed to cover the filing fees		FILING FEE AMOUNT
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	The invention was made by an agency of the United States Government or under a contract with an agency of the United States Government.
	No.
X	Yes, the name of the U.S. Government agency and the Government contract number are: U.S. Army Medical Research and Material Command, Grant No. DAMD17-00-1-0184, UCSD #9906948

Respectfully submitted,

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I certify that this provisional patent application cover sheet, provisional patent application and fee is being deposited on July 11, 2003, with the U.S. Postal Service as "Express Mail Post Office to Addressee" service under 37 C.F.R. 1.10 on the date indicated above and is addressed to: Commissioner for Patents, Mail Stop Provisional Patent Application, P.O. Box 1450, Alexandria, VA 22313-1450.

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Projects:
1. Design of optimized synthetic vaccines for cancer
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1996 to 6/2002: Assistant Project Scientist, Cancer Center,
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1. Cytotoxic T cell immunity against telomerase in humans
2. Cytotoxic T lymphocyte therapy for metastatic melanoma: Preclinical Development and Phase I/II Clinical Trial (in collaboration with *Johnson & Johnson*)
3. Peptide-loaded dendritic cells - a new approach for immunotherapy of cancer
4. Development of recombinant and synthetic anticancer vaccines
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